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Supporting Information

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Artemisone - A New, Highly Active Antimalarial Drug of the Artemisinin Class

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Supporting Information 1: Methods, Chemistry, Preparation of 10-Alkylamino Derivatives, Structure of Artemisone, Preparation of Radiolabelled Artemisone.

1. Methods

In vitro assays and rodent screens

In vitro antimalarial activity was assayed by inhibition of uptake by tritiated hypoxanthine,[1] and expressed as concentration at 50% inhibition (IC_{50}). *In vivo* assays were carried out with the 4-day suppressive test in mice infected with *P. berghei* or *P. yoelii*. Mice were treated sc or po from day of infection (d 0) to d +3. Results were evaluated as parasite counts in peripheral blood on d +4, and given as ED_{90} mg kg^{-1}.

Primate studies

Primate studies were approved by the AMI Animal Ethics Committee. Non-infected Saimiri monkeys received a single oral dose of artemisone or artesunate (30 mg kg^{-1}), heparinised blood was collected 1, 2, 3, 4 and 6 h after medication, and plasma was stored at -70 °C. At bioassay, plasma was serially diluted (1:2 to 1:8192) in a 96-well microplate and incubated with ring stages of *P. falciparum* K1 for 24-36 h. After incubation, blood films were made from each well and examined microscopically to determine the maximum dilution of drug plasma inhibiting >90% of parasites, relative to the control, from maturing from rings to schizonts. *In vivo* studies were commenced by inoculating 2 Aotus monkeys with blood containing Aotus-adapted *P. falciparum* FVO (resistant to chloroquine and antifolates). Blood from these monkeys was then used to infect 5 other malaria-naïve monkeys. Starting 6-9 d after inoculation, artesunate and artemisone were administered orally for 3 d at 10 mg $kg^{-1}d^{-1}$ (total 30 mg kg^{-1}) to 3 and 4 monkeys, respectively. Thick blood films were examined microscopically, usually twice a week after clearance of parasites, to assess response to treatment. Blood films were considered to be negative if no parasites were detected after examination of 100 microscopic fields (about 0.1 mL blood). Similar procedures were used for monkeys treated with artemisone combined with either mefloquine or amodiaquine.

Neurotoxicity assays

For *in vitro* assays, brain stem cells of fetal rats in the development stage E18-E19 were allowed to generate a permanent neuronal network during d 1-8. Compounds in DMSO were applied at 0.001, 0.01, 0.1, 1 and 10 μg mL^{-1} commencing at d 9 for a period of 7 d thereafter, with each compound being tested in triplicate with three different cell

preparations. Cytotoxicity was measured by the viability assay which determines activity of neuron-specific enolases, and neurotoxicity was assessed by the effect on the cytoskeleton. For *in vivo* pilot tolerability studies, compounds were administered in sesame or corn oil at 10 and 50 mg kg^{-1} via gavage to 2 x 2 10 wk old male Wistar rats. Animals were weighed daily, and were placed in an open field twice per day in order to observe for piloerection, respiratory abnormalities, posture, involuntary motor movement, stereotypy, gait abnormalities, vocalization, and other effects.

Metabolism and Pharmacokinetics

[^{2}H]- or [^{14}C]-Labelled artemisone was added to a suspension of human liver microsomes 452161 lot:22 (Gentest) at a protein concentration of 0.5 mg mL^{-1} in 1 mL pH 7.4 buffer (from 50 mM K_2HPO_4 and 1 mM EDTA) containing NADP (1 mM), glucose-6-phosphate (5 mM) and glucose-6-phosphate dehydrogenase (1.5 units), and the whole shaken at 37 °C and 1200 rpm. At 30 min, protein was precipitated by addition of acetonitrile (1 mL), the mixture centrifuged, and the supernatant concentrated to 70 µL. The metabolites from 10 such incubations were then separated by preparative HPLC using Latek-p 402 pumps with a Macherey and Nagel Nucleosil100 C18 HD, 250 x 10 mm 5 micron column, and UV or radiochemical detector, with mobile phases of 50 mM formic acid ammonium salt and acetonitrile under gradient elution at a flow rate of 4.0 mL min^{-1}. Metabolites were identified by LC-MS/MS and NMR spectroscopy, and by independent preparation of M1. The LC-UV-^{14}C-MS/MS comprised a capillary LC-System 1100 (Agilent), column oven (Mistral Slave, Spark) with 6-ways-tandem-microvalve (SunChrom) and an A/D-converter HP 35900 C (Hewlett-Packard). The MS was a Q-Star-Pulsar with API source operated in positive ion mode. ^{1}H and 2D-NMR spectra were recorded on a DRX 700 NMR spectrometer (Bruker) at 700 MHz and 25 °C in $CDCl_3$ solutions. Pharmacokinetics was performed by administering either unlabelled or labelled artemisone to female rats as a solution in ethanol, Solutol HS 15 and physiological saline, or as a suspension in aqueous tylose. Determination of unchanged artemisone was performed by protein precipitation with acetonitrile, and analysis by LC-MS/MS as described above. Limit of quantitation was 1 µg mL^{-1}.

The brief PK data is as follows. Peak plasma concentrations (C_{max}) in female rats following a single oral dose of artemisone at 3, 10 and 30 mg kg^{-1} were reached between 15 and 60 min. The corresponding C_{max}, area under the concentration-time curve (AUC) and elimination half lives ($t_{1/2}$) are 35, 230 and 850 µg L^{-1} , 22, 191 and 1008 µg.h L^{-1} and 0.18, 0.37 and 0.54 h. Based on intravenous AUC data, the bioavailability of artemisone ranges from 5-25%. The disproportionate increase in C_{max} and AUC values with increasing dose is suggestive of saturation kinetics. This is also apparent in 4-week toxicological studies in rats, in which on day 1, C_{max} values are 61, 320, and 3090 µg L^{-1} after oral administration of 5, 10, and 40 mg kg^{-1} respectively. With the exception of the apparent facile saturation of first pass metabolism, artemisone appears to have a roughly similar pharmacokinetic profile to other artemisinins. However, $t_{1/2}$ of artemisone in rats is at marked variance with the data in Fig. 3 (MS), which suggest $t_{1/2}$ in monkeys is about 2-3 h. The discrepancy arises through the nature of the *ex vivo* screen, which assays for total activity of artemisone plus the active metabolites **M1-M3** in monkey plasma.

Toxicity Studies

The 4-week sub-acute oral toxicity studies in non-rodent (Beagle dog) and rodent (rat) species, were carried out according to OECD guidelines for testing of chemicals No. 409, adopted Sept. 21, 1997, and No. 407, adopted July 21, 1995.

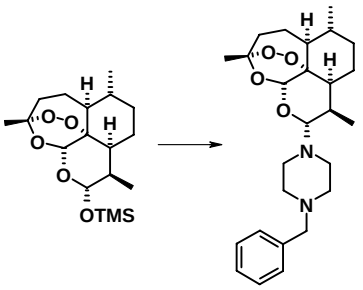
2. Chemistry

1. Preparation of 10-Alkylaminoartemisinins
General Experimental

The following solvents were dried and distilled prior to use: ethyl acetate ($MgSO_4$), hexane ($CaCl_2$), dichloromethane (CaH_2), triethylamine and thiomorpholine (CaH_2, distilled then stored over KOH), THF (Na/benzophenone), diethyl ether (Na/benzophenone), and toluene (Na/benzophenone). Thin layer chromatography was performed with Merck Kieselgel 60 F_{254} plates and visualised with ultra violet light (254 nm) and/or heating after treatment with 5% ammonium molybdate in 10% concentrated sulfuric acid. Column chromatography was performed with Merck silica gel 60 (0.04-0.063 mm). ^1H and ^{13}C NMR spectra were obtained with $CDCl_3$ solutions on Bruker ARX 300 and Varian Mercury 300 spectrometer operating at 300 and 75 MHz, respectively. Purity checks were run on a Varian Inova NMR Spectrometer at 750 MHz for ^1H spectra. Melting points were determined with a Leica Hot Stage DME E compound Microscope, and are corrected. Mass spectral data were obtained on a Finnigan TSQ 7000 Mass Spectrometer operating in CI mode, and on a API QSTAR high performance triple quadrupole time-of-flight mass spectrometer with electrospray ionization. Infrared spectra were recorded on a Perkin Elmer Spectrum One spectrometer. Polarimetry analyses were performed on a Perkin Elmer Model 241 polarimeter. Elemental analyses were obtained from MEDAC Ltd, Surrey, UK.

Compounds **9**, **10** and **11** were prepared according to the literature[2,3,4] or by the following representative procedures.

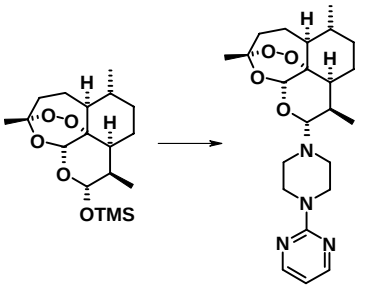
*Preparation of **9***



A cold (0 °C) stirred solution of 10α-(trimethylsiloxy)dihydroartemisinin (289.3 mg, 0.81 mmol) in dichloromethane (5 mL) was treated dropwise with bromo-trimethylsilane (107 μL, 124 mg, 0.81 mmol, 1.0 equiv.). After 30 min., this was transferred via cannula to a cold (0 °C) stirred solution of 1-benzylpiperazine (212.1 μL, 1.22 mmol, 1.5 equiv.) in dichloromethane (5 mL) and the resulting mixture was allowed to warm to room temperature overnight. The suspension was quenched with saturated aqueous $NaHCO_3$ solution. The organic layer was separated and dried ($MgSO_4$), and after evaporation of solvent, the residue was submitted to chromatography on silica gel with

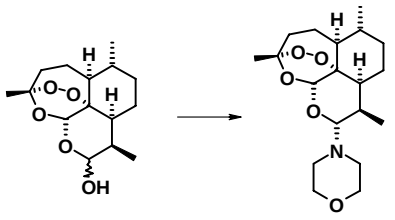
ethyl acetate-hexane (40:60) to give the product as a white solid (144.3 mg, 40%). Recrystallisation from dichloromethane-hexane gave needle-shaped crystals, m.p. 105-106 °C. $[\alpha]_D^{20}$ = + 10.3 (*c* = 0.909, $CHCl_3$). ^{1}H NMR: δ = 0.88 (d, *J* = 7.2 Hz, 3 H, 6-Me), 1.00-1.20 (m, 4 H), 1.20-1.70 (m, 9 H), 1.70-1.85 (m, 2 H), 1.85-2.02 (m, 1 H), 2.02-2.15 (m, 1 H), 2.30-2.70 (m, 7 H), 2.70-2.80 (m, 2 H), 3.06-3.11 (m, 2 H), 3.55 (d, *J* = 13.1 Hz, 1 H, benzylic-H), 3.62 (d, *J* = 13.1 Hz, 1 H, benzylic-H), 4.10 (d, *J* = 10.2 Hz, 1 H, H-10), 5.35 (s, 1 H, H-12), 7.30-7.43 (m, 5 H, Ar-H). ^{13}C NMR: δ = 13.4, 20.3, 21.6, 24.8, 26.0, 28.5, 34.3, 36.3, 37.4, 45.9, 51.7, 53.5, 63.1, 80.3, 90.4, 91.6, 103.8, 126.9, 128.1, 129.13, 138.3. IR (film): ν_{max} = 694, 738, 824, 852, 880, 924, 942, 986, 1016, 1042, 1062, 1114, 1132, 1204, 1270, 1294, 1344, 1376, 1454, 1494, 2802, 2860, 2920, 2954 cm^{-1}. MS (CI, CH_4): *m/z* (%) = 443 (10) [M^++1]. $C_{26}H_{38}N_2O_4$ (442.60): calcd. C 70.56, H 8.65, N 6.33, found C 70.24, H 8.67, N 6.28.

*Preparation of **10***



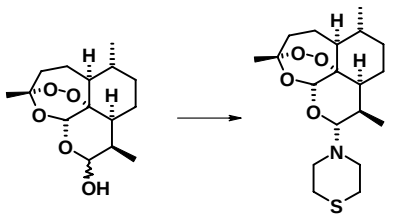
A cold (0 °C) stirred solution of 10α-(trimethylsiloxy)dihydroartemisinin (350 mg, 0.98 mmol) in dichloromethane (5 mL) was treated dropwise with bromotrimethylsilane (136 μL, 158 mg, 1.03 mmol, 1.05 equiv.). After 30 min., this was transferred via cannula to a cold (0 °C) stirred solution of 1-(2-pyrimidyl)piperazine (0.32 g, 1.97 mmol, 2.0 equiv.) in dichloromethane (5 mL) and the resulting mixture was allowed to warm to room temperature overnight. The suspension was quenched with saturated aqueous $NaHCO_3$ solution. The organic layer was separated and dried ($MgSO_4$). Filtration and evaporation of filtrate gave a residue, which on chromatography with ethyl acetate-hexane (20:80) gave the product as a fine microcrystalline powder. Recrystallization from ethyl acetate- hexane gave **10** as fine needles (184 mg, 44%), m.p. 147.1-147.5 °C. $[\alpha]_D^{20}$ = +14.30 (*c* = 0.86, $CHCl_3$). ^{1}H NMR: δ = 0.85 (d, *J* = 7.16 Hz, 3 H, 9-Me), 0.94 (d, *J* = 6.11 Hz, 3 H, 6-Me), 1.17-1.33 (m, 1 H), 1.34 (s, 3 H, 3-Me), 1.37-2.02 (m, 8 H), 2.27-2.38 (m, 1 H), 2.59-2.75 (m, 4 H, 2 × NCH_2), 3.01-3.08 (m, 2 H), 3.72-3.87 (m, 4 H, 2 × NCH_2), 4.05 (d, *J* = 10.23 Hz, 1 H, H-10), 5.27 (s, 1 H, H-12), 6.43 (t, *J* = 4.82 Hz, *para*-Ph, 1 H), 8.27 (d, *J* = 4.83 Hz, 2 H, *ortho*-Ph). ^{13}C NMR: δ = 14.22, 20.97, 22.34, 25.44, 26.66, 29.20, 34.99, 37.02, 38.07, 44.76, 46.56, 47.95, 52.44, 80.99, 91.49, 92.34, 104.56, 110.17, 158.31, 162.39. IR (film): ν_{max} = 640, 694, 742, 798, 826, 852, 880, 926, 940, 980, 1022, 1044, 1060, 1102, 1114, 1132, 1160, 1186, 1208, 1268, 1308, 1376, 1396, 1430, 1438, 1452, 1502, 1546, 1588, 2854, 2870, 2918, 2948, 2970, 2988 cm^{-1}. MS (CI, CH_4): *m/z* (%) = 431 (33) [MH^+], 432 (5) [MH^+, ^{13}C]. $C_{23}H_{34}N_4O_4$ (430.55): calcd. C 64.16, H 7.96, N 13.01; found C 64.09, H 8.07, N 12.86.

Preparation of ***11***



According to the forgeoing procedure, the product was obtained from DHA 10α-TMS ether (214 mg, 0.60 mmol), trimethylsilyl bromide (79.3 μL, 92 mg, 0.6 mmol, 1.0 equiv.) and morpholine (105 μL, 1.20 mmol, 2.0 equiv.) and chromatography over silica gel with ethyl acetate-hexane (20:80).as a white solid (59.5 mg, 61%)T, m.p. 121.2 °C. $[\alpha]_D^{20}$ = +15.3 (*c* = 0.30, $CHCl_3$). ^{1}H NMR: δ = 0.83 (d, *J* = 7.18 Hz, 3 H, 9-Me), 0.96 (d, *J* = 6.14 Hz, 3 H, 6-Me), 1.41 (s, 3 H, 3-Me), 1.00-2.06 (m, 10 H), 2.31-2.41 (m, 1 H), 2.53-2.61 (m, 1 H, H-9), 2.64-2.71 (m, 2 H, morpholino CH_2), 2.96-3.03 (m, 2 H, morpholino CH_2), 3.63-3.76 (m, 4 H, morpholino CH_2), 3.99 (d, *J* = 10.23 Hz, 1 H, H-10), 5.29 (s, 1 H, H-12). ^{13}C NMR: δ = 13.25, 20.14, 21.50, 24.62, 25.84, 28.04, 34.14, 36.16, 37.25, 45.66, 47.52, 51.57, 67.25, 80.16, 90.51, 91.48, 103.74. IR (film): ν_{max} = 744, 826, 846, 880, 930, 984, 1056, 1110, 1158, 1202, 1258, 1294, 1376, 1450, 2850, 2924 cm^{-1}. MS (EI) *m/z* (%) = 88 (24), 116 (100), 127 (32), 163 (14), 209 (12), 221 (16), 236 (4), 294 (4), 353 (6) [M^+]. $C_{19}H_{31}NO_5$ (353.46): calcd. C 64.56, H 8.84, N 3.96; found C 64.67, H 9.10, N 3.90.

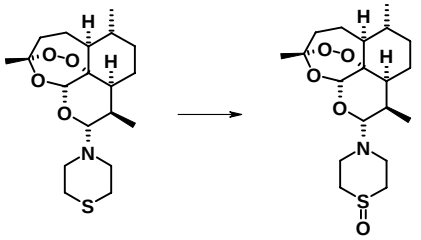
Preparation of ***12***



Chlorotrimethylsilane (0.279 mL, 2.2 mmol) was added to a stirred solution of dihydroartemisinin (**2**, 284 mg, 1.00 mmol) and sodium bromide (114 mg, 1.1 mmol) in toluene (1.0 mL) at room temperature. After 15 min, neat thiomorpholine (251 μL, 2.5 mmol) was added to the mixture in a single stroke. The solution was stirred vigorously for another 15 min. Work-up was performed by adding saturated aqueous sodium bicarbonate (5 mL) and diluting the mixture with dichloromethane (10 mL). The organic layer was collected, and dried (Na_2SO_4), and after filtration, solvent was removed under reduced pressure. The residue was purified by chromatography with ethyl acetate-hexanes (8:92) as eluent to give **12** as a white crystalline solid. Recrystallization from methanol gave needles (214 mg, 58%), m.p. 152.5-153.0 °C, $[\alpha]_D^{20}$ = +17° (*c* = 2.1, $CHCl_3$). ^{1}H NMR: δ = 0.76 (d, *J* = 7.18 Hz, 3 H, 6-Me), 0.91 (d, *J* = 6.14 Hz, 3 H, 9-Me), 0.90-1.04 (m, 1 H), 1.36 (s, 3 H, 3-Me), 1.14-1.52 (m, 5 H), 1.63-1.70 (m, 2 H), 1.78-1.86 (m, 1 H), 1.93-2.01 (m, 1 H), 2.25-2.36 (m, 1 H), 2.53-2.68 (m, 5 H), 2.85-2.93 (m,

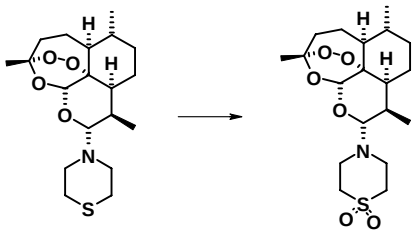
2 H), 3.20-3.28 (m, 2 H), 3.93 (d, *J* = 10.21 Hz, 1 H, H-10), 5.23 (s, 1 H, H-12). ^{13}C NMR: δ = 13.41, 20.15, 21.44, 24.59, 25.84, 28.15, 34.12, 36.14, 37.19, 45.66, 50.39, 51.54, 80.11, 91.42, 92.28, 103.70. IR (film): ν_{max} = 756, 828, 850, 880, 926, 940, 988, 1018, 1038, 1056, 1100, 1130, 1154, 1184, 1198, 1226, 1278, 1326, 1376, 1418, 1454, 2872, 2924 cm^{-1}. MS (CI, NH_3): *m/z* (%) = 310 (10), 324 (70), 370 (100) [M^++1]. $C_{19}H_{31}NO_4S$ (369.53): calcd. C 61.76, H 8.46, N, 3.79; found C 62.04, H 8.39, N 3.65.

*Oxidation of **12** to **13***



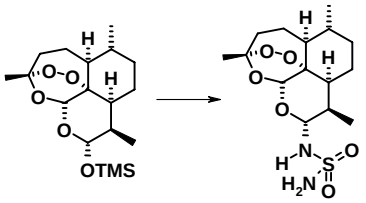
Activity of *m*-chloroperbenzoic acid (Aldrich) was estimated by treating a solution of sulfide **12** in diethyl ether containing potassium carbonate at 0 °C with approximately one equivalent of peracid, and in the presence of an NMR standard (1,3,5-trimethoxybenzene), analyzing the ratio of starting material **12** to product **13** in the crude reaction mixture. In this way, the commercial peracid was estimated to contain 71% active peracid. *m*-Chloroperbenzoic acid (71% *m*-chloroperbenzoic acid, 65.7 mg, containing 46.65 mg active peracid, 0.27 mmol, 1.0 equiv) was added all at once to a stirred solution of sulfide **12** (100 mg, 0.27 mmol) in diethyl ether (7 mL) containing potassium carbonate (187.4 mg, 1.36 mmol, 5.0 equiv) at 0 °C. The mixture was allowed to stir for 5 hours at 0 °C, and was then quenched with saturated sodium bicarbonate (15 mL). The organic layer was separated and it was washed with more saturated sodium bicarbonate (15 mL). The aqueous layer was extracted once with diethyl ether (15 mL), and the organic extracts were combined, and washed with brine (10 mL) and then dried ($MgSO_4$). The solvent was evaporated to give the crude product as a colourless gum. The yield of sulfoxide **13** (72%) was calculated from ^{1}H NMR spectroscopy based on the addition of standard 1,3,5-trimethoxybenzene. Direct crystallization of the crude product from a larger scale reaction commencing with sulfide **12** (2.5 g, 6.78 mmol) from iso-propanol gave the sulfoxide as a white micro-crystalline solid (1.84 g, 71%), m.p 158.6-161.1 °C; $[\alpha]_D^{21}$ = +13.94 (*c* = 0.76, $CHCl_3$). ^{1}H NMR: δ = 0.79 (d, *J* = 7.33 Hz, 3 H, 9-Me), 0.94 (d, *J* = 6.16 Hz, 3 H, 6-Me), 0.97-1.10 (m, 1 H), 1.20-1.51 (m, 2 H), 1.53 (s, 3 H, 3-Me), 1.54-1.58 (m, 2 H), 1.66-1.76 (m, 3 H), 1.82-1.86 (m, 1 H), 1.87-2.03 (m, 1 H), 2.28-2.39 (ddd, *J* = 4.10, 14.37, 18.5 Hz, 1 H), 2.52-2.64 (m, 1 H, morpholino CH_2), 2.71-2.81 (m, 2 H, morpholino CH_2), 2.88-2.96 (m, 1 H, morpholino CH_2), 2.95-3.13 (m, 3 H, morpholino CH_2 and DHA CH), 3.69-3.46 (m, 2 H, morpholino CH_2), 4.09 (d, *J* = 10.26 Hz, 1 H, H-10), 5.27 (s, 1 H, H-12). ^{13}C NMR: δ = 13.50, 20.22, 21.59, 24.74, 25.94, 28.82, 34.21, 36.23, 37.40, 39.67, 41.90, 45.70, 47.03, 47.15, 51.59, 80.21, 91.39, 91.84, 104.05. IR (film): ν_{max} = 926, 938, 1014, 1040, 1377, 1450, 2871, 2925 cm^{-1}. MS (CI, CH_4): *m/z* (%) = 385 (6) [M^+], 386 (100) [MH^+], 387 (24) [MH^+, ^{13}C], 388 (9) [MH^+, 2 x ^{13}C], 414 (15) [MH + C_2H_8]$^+$]. $C_{19}H_{31}NO_5S$ (369.53): calcd. C 59.20, H 8.10, N 3.63; found C 59.48, H 8.15, N 3.38.

*Preparation of Artemisone **14***



To a solution of compound **12** (388 mg, 1.05 mmol) in dichloromethane (10 mL) at room temperature under nitrogen was added *N*-methylmorpholine-*N*-oxide (369 mg, 3.15 mmol), powdered molecular sieve (525 mg, 4 Å), and tetrapropylammonium perruthenate (18.5 mg). The mixture was stirred at room temperature overnight after which it was filtered through a pad of silica and the residue was washed with ethyl acetate (3 x 15 mL). The filtrate was concentrated in vacuo. The residue was then purified by flash chromatography (silica gel; 35% ethyl acetate/hexanes) to give artemisone (421 mg, 100%), which was shown by ^{1}H NMR spectroscopy at 750 MHz to be pure. Isomeric impurities could not be detected. It crystallized from ethyl-acetate-hexane as needles, m.p. 152.3-152.7 °C; $[\alpha]_D^{20}$ +13° (c 0.035/$CHCl_3$); ν_{max} (film) 2928, 2872, 1454, 1378, 1308, 1270, 1228, 1198, 1124, 1040, 1018, 976, 940, 878, 846, 826, 752, 704, 666; δ_H: 5.27 (1H, s, H-12), 4.21 (1H, d, J= 10.30 Hz, H-10), 3.18-3.46 (8H, m), 2.54-2.62 (1H, m), 2.28-2.36 (1H, m), 1.20-2.02 (9H, m), 1.35 (3H, s, H-14), 0.92-1.06 (1H, m), 0.93 (3H, d, J= 5.99 Hz, H-15), 0.78 (3H, d, J= 7.13 Hz, H-16); δ_C: 174.20, 104.09, 91.92, 90.84, 90.04, 51.74, 51.27, 46.88, 45.46, 37.29, 36.02, 34.04, 28.91, 25.76, 24.66, 21.45, 20.10,13.31; m/z (CI, NH_3) 402 (M^++1, 100), 373 (30), 356 (64), 342 (16), 356 (20); Anal. Calcd. for $C_{19}H_{31}NO_6S$: C, 56.84; H, 7.78; N, 3.49; found: C, 56.83; H, 7.82; N, 3.37.

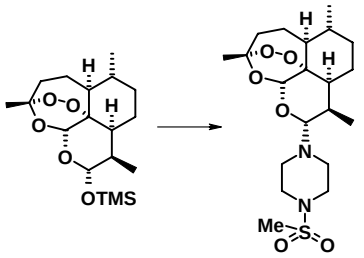
*Preparation of **15***



Trimethylsilyl bromide (0.16 g, 0.14 ml, 1.05 mmol) was added dropwise to a cold (0 °C) stirred solution of 10α-(trimethylsiloxy)dihydroartemisinin (356 mg, 1.0 mmol) in dichloromethane (5 mL). After 15 min, a solution of sulfamide (0.19 g, 2.0 mmol) in dry acetone (6 mL) was added. After 1.5 h, the reaction was quenched with saturated aqueous $NaHCO_3$ (10 mL) and extracted with diethyl ether (3 x 10 mL). The organic extracts were combined and dried ($MgSO_4$). Filtration and evaporation of filtrate gave a dark green solid which was purified by column chromatography on silica with ethyl acetate-hexanes (40:60) as eluent to give the product **15** as a white powder. This was recrystallized from ethyl acetate to give fine needles (260 mg, 70%), m.p. 168.0-168.7 °C; $[\alpha]_D^{22}$ = +16.76° (*c* = 0.68, MeOH). ^{1}H NMR (d_6-DMSO): δ = 0.78 (d, *J* = 7.11 Hz, 3 H, 6-Me), 0.89 (d, *J* = 6.23 Hz, 3 H, 9-Me), 0.94-1.10 (m, 1 H), 1.12-1.20 (m, 1 H), 1.28

(s, 3 H, 3-Me), 1.31-1.51 (m, 4 H), 1.61-1.64 (m, 2 H), 1.77-1.82 (m, 1 H), 1.96-2.00 (m, 1 H), 2.12-2.31 (m, 2 H), 4.58 (dd, *J* = 9.2, 9.2 Hz, 1 H, H-10), 5.37 (s, 1 H, H-12), 6.44 (s, 2 H, NH_2, D_2O exchanged), 7.79 (d, *J* = 8.63 Hz, 1 H, NH, D_2O exchanged). ^{13}C NMR: δ = 13.60, 20.46, 21.24, 24.57, 25.66, 31.56, 33.66, 36.01, 36.34, 45.14, 51.36, 79.98, 80.47, 90.61, 103.31. IR (film): ν_{max} = 1024, 1128, 1147, 1308, 1323, 1375, 1456, 1631, 2880, 2934, 2959, 3226, 3387 cm^{-1}. MS (CI, CH_4): *m/z* (%) = 363 (7) [MH^+], 364 (1) [MH^+, ^{13}C]. $C_{15}H_{26}N_2O_6S$ (362.45): calcd. C 49.71, H 7.23, N 7.73; found C 49.59, H 7.29, N 7.58.

*Preparation of **16***

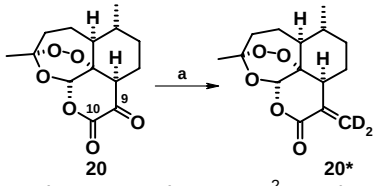


The product was obtained from DHA 10α-TMS ether (574 mg, 1.61 mmol), trimethylsilyl bromide (0.26 g, 0.22 mL, 1.69 mmol, 1.05 equiv.) and methanesulfonyl-piperazine (529 mg, 3.23 mmol, 2.0 equiv.) according to the literature procedure[2,3,4] as a white powder. Recrystallisation with ethyl acetate-hexane gave **16** as white needles (319.9 mg, 46%), m.p. 149.9-150.0 °C. $[\alpha]_D^{22}$ = +10.96 (*c* = 0.68, $CHCl_3$). ^1H NMR: δ = 0.81 (d, *J* = 7.18 Hz, 3 H, 6-Me), 0.95 (d, *J* = 6.09 Hz, 3 H, 9-Me), 0.98-1.08 (m, 1 H), 1.18-1.36 (m, 2 H), 1.38 (s, 3 H, 3-Me), 1.40-1.59 (m, 3 H), 1.68-1.74 (m, 2 H), 1.83-1.90 (m, 1 H), 1.97-2.04 (m, 1 H), 2.29-2.40 (m, 1 H), 2.54-2.61 (m, 1 H), 2.76-2.83 (m, 5 H, morpholino CH_2, SO_2Me), 3.05-3.12 (m, 2 H, morpholino CH_2), 3.17-3.30 (m, 4 H, 2 x morpholino CH_2), 4.06 (d, *J* = 10.26 Hz, 1 H, H-10), 5.28 (s, 1 H, H-12). ^{13}C NMR: δ = 13.40, 20.21, 21.54, 24.69, 25.90, 28.41, 34.18, 34.40, 36.21, 37.32, 45.69, 46.06, 46.91, 51.60, 80.17, 90.56, 91.47, 103.91. IR (film): ν_{max} = 756, 778, 880, 926, 959, 1027, 1042, 1131, 1163, 1205, 1264, 1325, 1342, 1377, 1454, 2872, 2928 cm^{-1}. MS (CI, CH_4): *m/z* (%) = 432 (6) [MH^+, ^{13}C], 431 (28) [MH^+]. $C_{20}H_{34}N_2O_6S$ (430.57): calcd. C 55.79, H 7.96, N 6.50; found C 55.78, H 8.00, N 6.43.

3. Preparation of 9-[C^2H_3]-artemisone 14*

a-Discussion

a. *Conversion of dicarbonyl compound **20** into 9-[C^2H_2]-artemisitene*: Reaction of the dicarbonyl compound **20** with [C^2H_2]-methylidene triphenylphosphorane, generated from [C^2H_3]-methyltriphenylphosphonium bromide (containing 95+ atom % ^2H) by treatment with *n*-butyllithium in THF at -78 °C followed by warming to room temperature gave labelled artemisitene **20*** in 48% yield.



Scheme 1: Wittig reaction on dicarbonyl compound **20**: a, $C^2H_3PPh_3Br$ (1.1 equiv.), *n*-C_4H_9Li (1.1 equiv.), N_2, THF, -78 °C then dicarbonyl compound **20** (1.0 equiv.) -78-20 °C, 20 h, then NH_4Cl (aq.) quench (48%).

The NMR spectra of each of artemisitene and the 9-[C^2H_2]- compound is given in Figs. 1a and 1b. It can be seen that the exocyclic methylidene protons, whose signals appear at δ 5.67 ppm and at 5.99 ppm, in artemisitene, do not appear in the 9-[C^2H_2]-artemisitene.

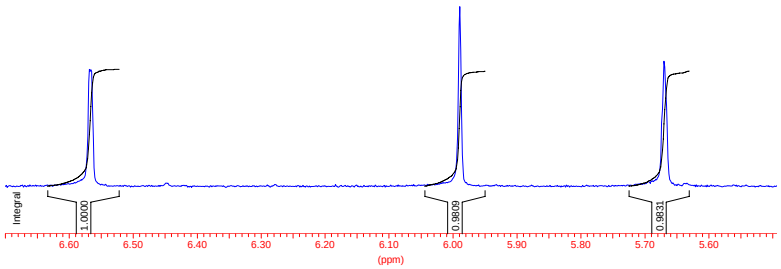


Figure 1a: ^1H NMR spectrum of artemisitene (300 MHz, $CDCl_3$) in region δ 5.5-6.7 ppm illustrating absorptions due to methylidene protons (δ = 5.99. 5.67 ppm) and H-12 (δ = 6.59).

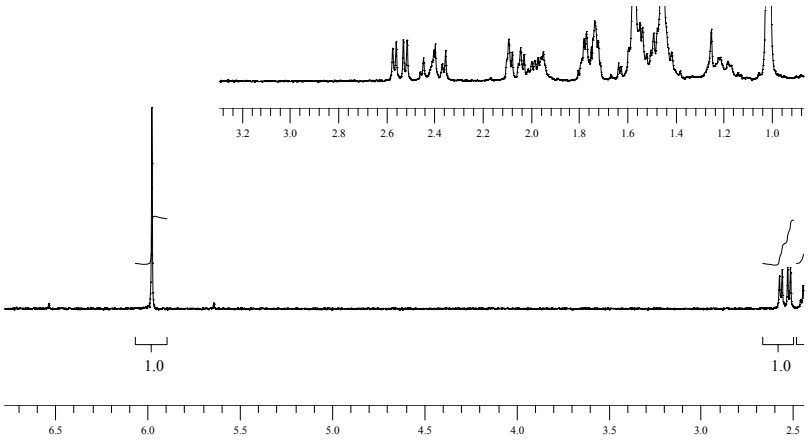
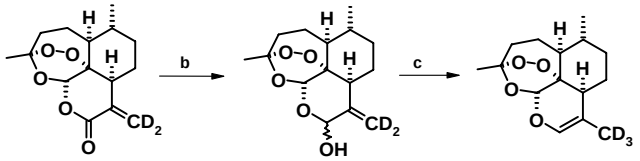


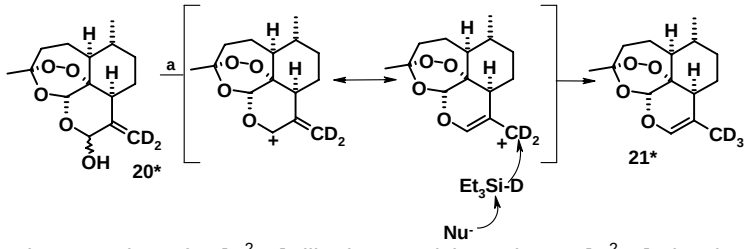
Figure 1b: ^1H NMR spectrum of [9-C^2H_2]-artemisitene **20*** (300 MHz, $CDCl_3$) in region δ 5.5-6.7 ppm illustrating absorption due to H-12 (δ = 6.00 ppm) (inset: upfield part of spectrum).

b. *Reduction of 9-[C^2H_2]-artemisitene*: Treatment of 9-[C^2H_2]-artemisitene with an excess of diisobutylaluminium hydride in dichloromethane at -78 ºC gave 9-[C^2H_2]-dihydroartemisitene, as a mixture of epimers in quantitative yield. This mixture was not purified, but submitted directly to the next step.



Scheme 2. Reduction of artemisitene: b, *i*-Bu_2AlH (1.9 equiv.), CH_2Cl_2, -78 ºC, 2 h, then quench, >98% crude yield; 54% recrystallized; c: Et_3SiH or Et_3SiD (4.1 equiv.), $BF_3.OEt_2$ (1.15 equiv.), CH_2Cl_2 -78 ºC, 1.5 h, then quench *or* Et_3SiH or Et_3SiD (4.1 equiv.), TFA (2.0 equiv.), -78 °C, 4 h, quench, 98% crude yield, 40% purified yield.

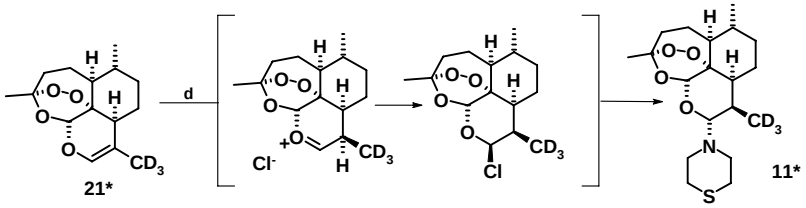
c. *Ionic reduction of 9-[C^2H_2]-dihydroartemisitene*: The next step was to carry out the ionic reduction of the allylic alcohol. This type of reaction has not been attempted before. In the presence of an acid and a hydride donor, a stabilized cation should be generated, which may add hydride at the least substituted exocyclic site (Scheme 3).



Scheme 3: Proposed conversion of 9-[C^2H_2]-dihydroartemisitene into 9-[C^2H_3]-glycal a: Et_3SiD (excess), CF_3COOH ($Nu^- = CF_3COO^-$), CH_2Cl_2 then quench.

The reduction of 9-[C^2H_2]-dihydroartemisitene **20*** was carried out with ^2H-triethylsilane (triethylsilane-D, 97 atom % D) and trifluoroacetic acid to give 9-[C^2H_3]-glycal **21*** in 40% overall yield. The ^1H NMR spectrum indicated clean formation of the labelled compound; the methyl signal at δ 1.60 ppm in the glycal due to the methyl group was not present in the spectrum of the deuterated compound.

d. *Conversion of 9-[C^2H_3]-glycal into 9-[C^2H_3] thiomorpholine derivative*: With the reconstituted glycal **21*** in hand, it was now possible to convert this compound into the thiomorpholino derivative **12***.



Scheme 4. Preparation of thiomorpholino derivative **11*** from glycal **21***. d. HCl, CH_2Cl_2, 0 °C, then thiomorpholine (1.1 equiv.), triethylamine (1.0 equiv.), 0 °C, 45 min, then brine, 50%.

The 9-[C^2H_3]-glycal **21***, in accord with the chemistry of anhydro sugars,[5] was treated with dry HCl in toluene or dichloromethane to produce the β-chloride intermediate *in situ*, identified by ^1H NMR spectroscopic examination of reactions conducted with unlabelled reagents in $CDCl_3$ (Scheme 4) This intermediate was treated with thiomorpholine in the presence of triethylamine to give the product **11*** (56%). The

glycal **21*** (22%) was also formed in this reaction. This is very likely to arise via a competing elimination of the axial chloride when it is treated with the basic nucleophile. It was collected and recycled. No attempt was made to purify the 9-[C^2H_3]-thiomorpholine derivative. The ^{1}H NMR spectra of the thiomorpholine derivative **11** and the 9-[C^2H_3] thiomorpholine derivative **11*** are given below (Figs. 2a and 2b). The signal due to the C9 methyl group at δ 0.78 ppm is not present in the deuterated derivative.

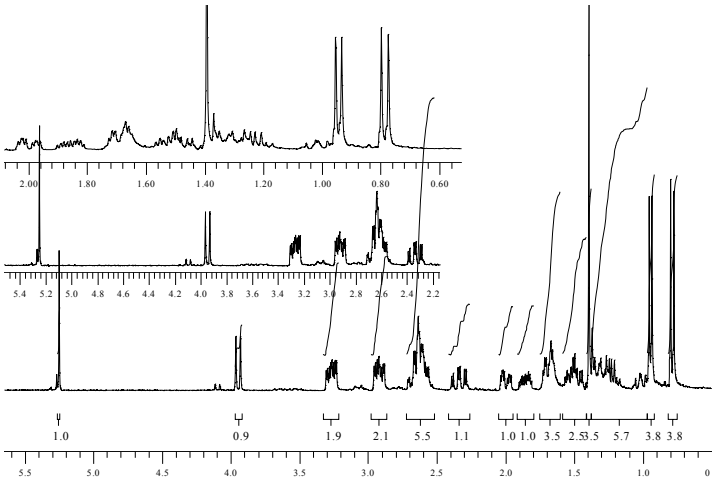


Figure 2a. ^{1}H NMR spectrum of thiomorpholine derivative. The signal due to the methyl group at C9 appears at δ 0.78 ppm as a doublet with J = 10.2 Hz.

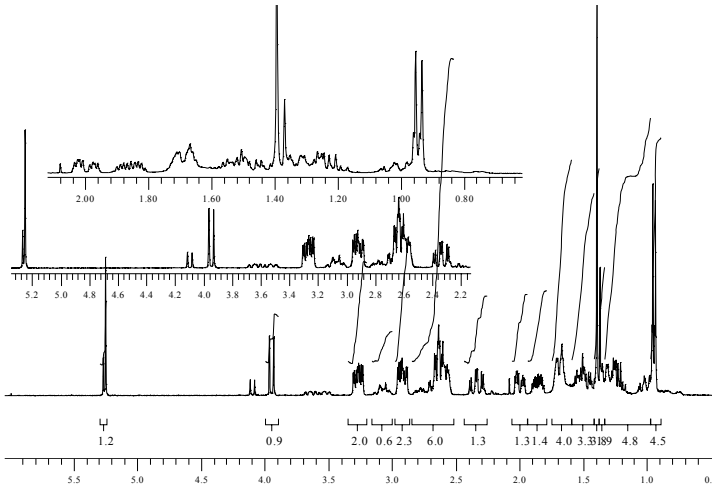
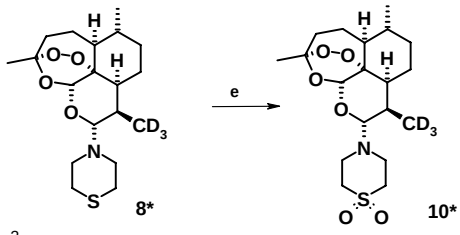


Figure 2b. ^{1}H NMR spectrum of 9-[C^2H_3] thiomorpholine derivative.

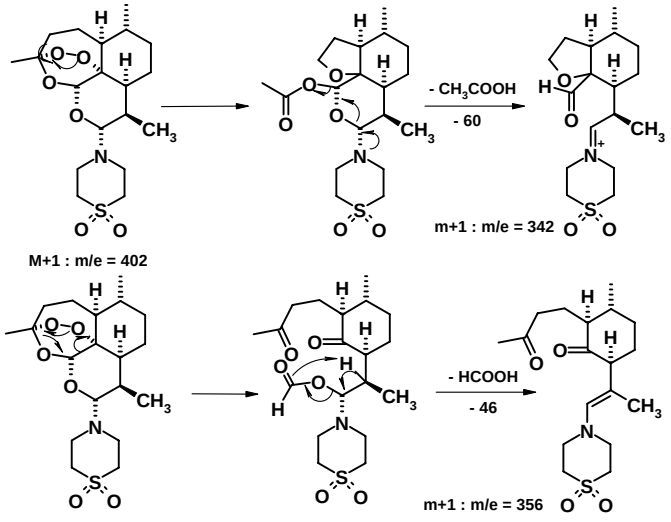
e. *Conversion of 9-[C^2H_3] thiomorpholine derivative into 9-[C^2H_3]-artemisone*: The final step involves the conversion of the 9-[C^2H_3]-thiomorpholine adduct into 9-[C^2H_3]-artemisone by oxidation of the sulfide to the sulfone. Many reagents may be used for the reaction with the unlabelled compounds, but best was found to be *m*-chloroperbenzoic acid, because it is a rapid oxidation, which is important for the radiolabelled compound. Oxidation of 9-[C^2H_3] thiomorpholine with two equivalents of the reagent in diethyl ether

containing an excess of potassium carbonate derivative gave 9-[C^2H_3]-artemisone in 72% yield.



Scheme 5. Conversion of 9-[C^2H_3] thiomorpholine derivative into 9-[C^2H_3]-artemisone. **e.** *m*-chloroperbenzoic acid, K_2CO_3, Et_2O, 0 °C, 72%.

f. *9-[C^2H_3]-artemisone*: The final product was analyzed by ^{1}H NMR spectroscopy and by mass spectrometry. The ^{1}H NMR spectra of each of artemisone and 9-[C^2H_3]-artemisone are given in Figs. 3a and 3b. It is seen that the signal due to 9-CH_3 group in artemisone at δ 0.78 ppm is absent in 9-[C^2H_3]-artemisone. Also, the signal due to H-9 at 2.54-2.62 changed from a multiplet for artemisone to a doublet of doublets for 9-[C^2H_3]-artemisone, because in the case of 9-[C^2H_3]-artemisone, there is only coupling between H-9 and each of H-8a and H-10. The electrospray ionization mass spectrum of 9-[C^2H_3]-artemisone is given in Fig. 3c. The protonated molecular ion corresponding to $C_{19}H_{29}D_3NO_6S^+$ appears at 405.2129, for a calculated mass of 405.2136. In Figs 4a and 4b are shown the mass spectra (CI, CH_4), of the 9-[C^2H_3]-artemisone and 9-[CH_3]-artemisone respectively. The mass of the protonated molecular ion of 9-[C^2H_3]-artemisone is 405.1 and the mass of the protonated molecular ion of 9-[CH_3]-artemisone is 402.1 with the unit differing by 3. This difference appears in all the other fragmentation peaks at the mass spectra. The proposed pathway is shown in Scheme 6.



Scheme 6. The proposed MS fragmentation pathways of artemisone

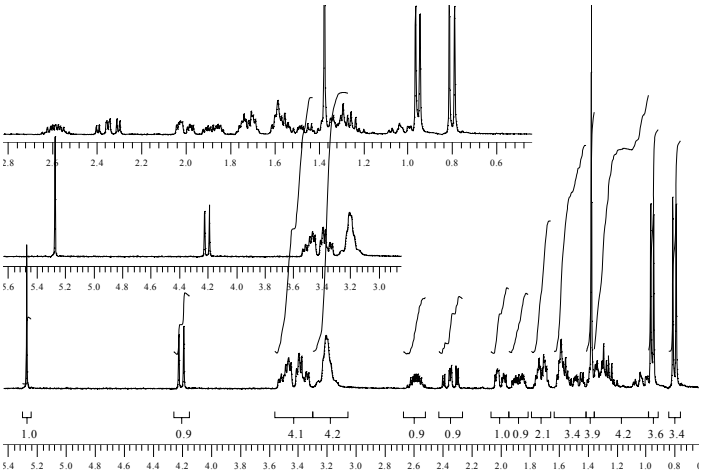


Figure 3a. ^{1}H NMR spectrum of artemisone (300 MHz, $CDCl_3$). Signal due to 9-CH_3 is at δ= 0.78 ppm, d, *J* = 7.1 Hz. Other assignments δ = 0.93 (d, *J* = 5.99 Hz, 3 H, 6-Me), 1.35 (s, 3 H, 3-Me), 3.18-3.46 (m, 8 H thiomorpholine), 4.21 (d, *J* = 10.30 Hz, 1 H, H-10), 5.27 (s, 1 H, H-12).

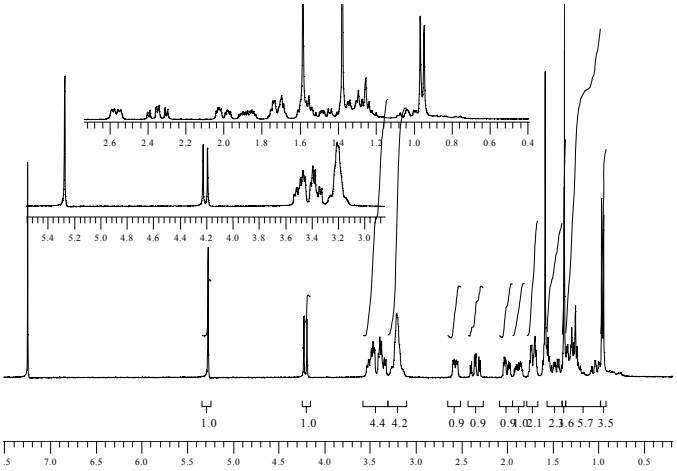


Figure 3b. ^{1}H NMR spectrum of 9-[$C^{2}H_3$]-artemisone (300 MHz, $CDCl_3$). Assignments δ = 0.97-0.95 (3H, d, *J* = 6.3 Hz, 6 Me), 1.38 (3H, s, 3 Me), 2.59-2.54 (1H, dd, J = 10.5 Hz, 4.8 Hz, H-9), 3.26-3.12 (4H, m), 3.53-3.33 (4H, m), 4.22-4.19 (1H, d, J = 10.2 Hz, H-10), 5.27 (1H, s, H-12).

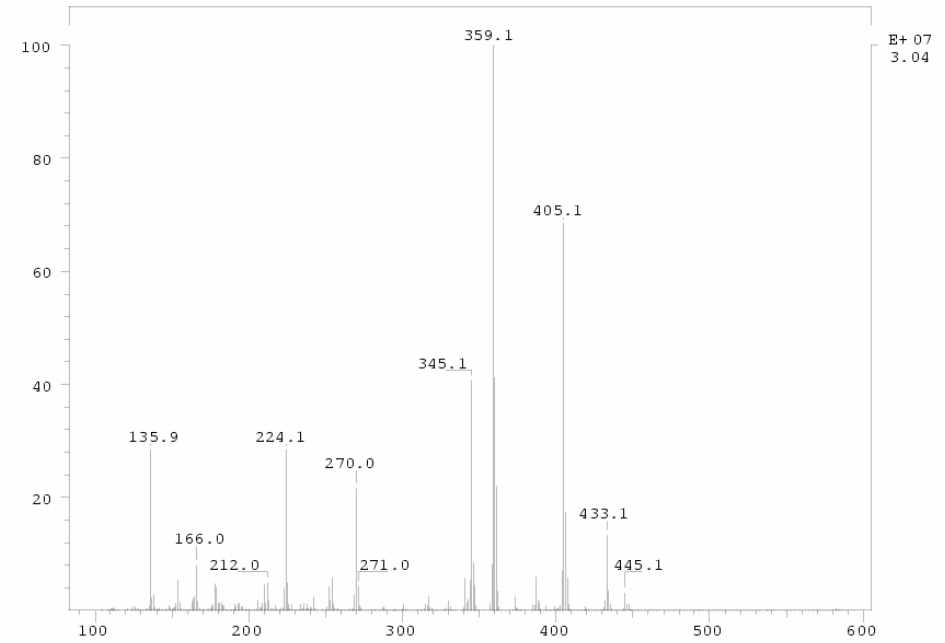


Figure 4a. The mass spectrum (CI, CH_4) of 9-CD_3 artemisone

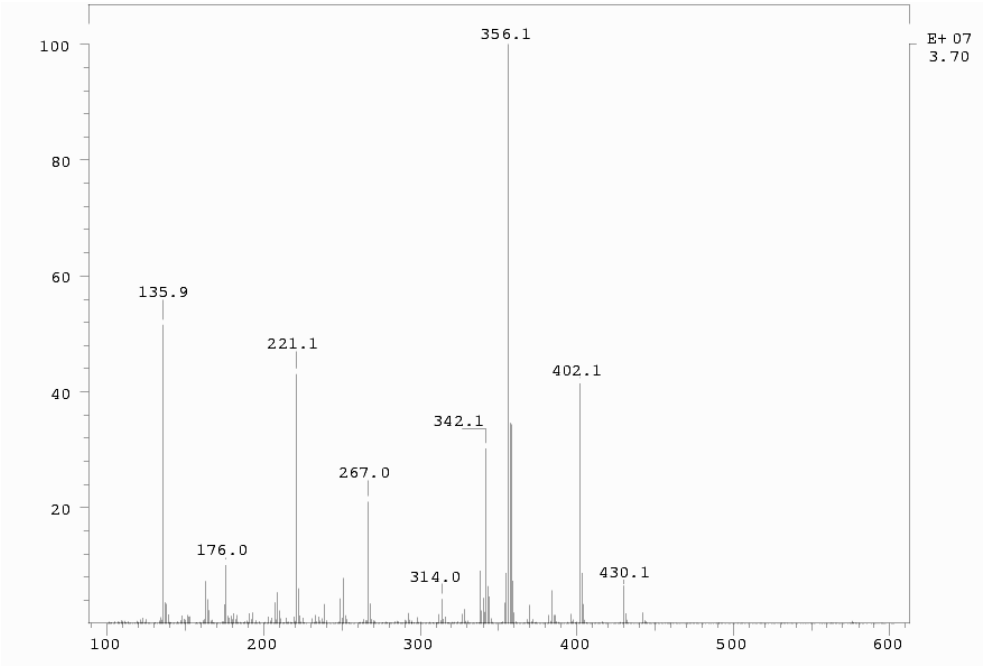


Figure 4b. The mass spectrum (CI, CH_4) of artemisone

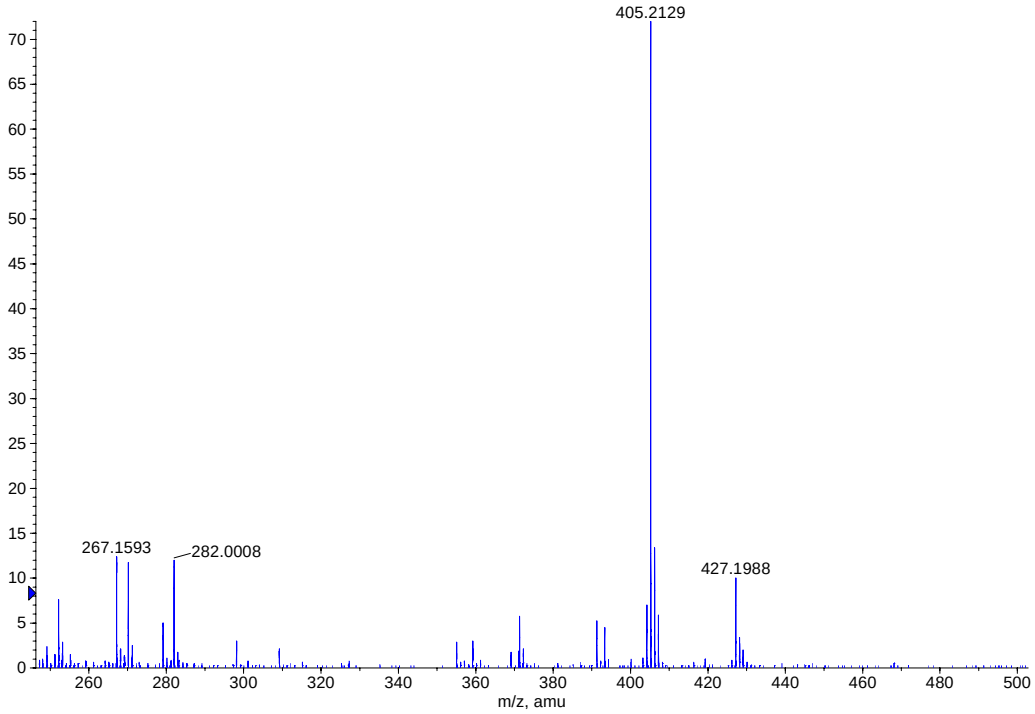
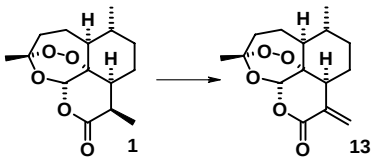


Figure 4c. The mass spectrum (ESI) for the accurate mass measurement of 9-CD_3 artemisone, showing the mass (M+1) of 405.2129, the calculated mass (M+1) is 405.2136.

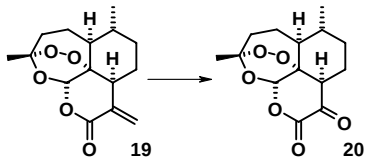
b-Experimental

*Preparation of Artemisitene from Artemisinin **1***[6]



A solution of artemisinin **1** (564 mg, 2.0 mmol) was slowly added to a solution of lithium diisopropylamide (2.2 mmol) in tetrahydrofuran (THF, 5 mL) at –78 °C, and the resulting mixture was stirred thereafter at –78 °C for 15 min. A solution of phenylselenenide bromide (519 mg, 2.2 mmol) in THF (10 mL) was added, and the mixture was stirred for a further 15 min. The reaction mixture was treated sequentially with glacial acetic acid (0.3 mL) and a solution of 30% aqueous hydrogen peroxide (1.5 mL). The reaction temperature was raised to 0 °C, and the mixture was stirred for 30 min. Water (50 mL) was added, and the mixture was extracted with dichloromethane (3 x 20 mL). The organic extracts were combined, and the organic layer was washed with saturated aqueous sodium bicarbonate, and then with water, and dried ($MgSO_4$). The solvent was evaporated under reduced pressure, and the residue was purified by column chromatography with ethyl acetate-hexane (20:80) to give artemisitene **19** (369 mg, 66%).

*Preparation of 9-Desmethyl-9-oxoartemisinin **20***



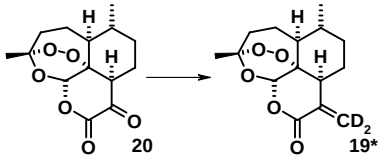
Method 1: Ozone[7]

Ozone, generated by passage of compressed air through an OREC ozone generator (model PHF200A106-119), was passed for 6 h through a solution of artemisitene **20** (5 g, 17.86 mmol) in dichloromethane (100 mL) cooled by immersion in a dry ice-acetone bath. When the solution became pale blue, it was allowed to warm to room temperature and purged with nitrogen for 10 min. Diethyl sulfide (12.5 mL) was added, and the solution was stirred at room temperature for 1 h. The solvent was evaporated under reduced pressure to leave a residual yellow oil, which was kept overnight under vacuum. The resulting solid was recrystallized directly from dichloromethane/diethyl ether to give large lustrous pale yellow rectangular crystals of **20** (3.332 g, 66%), m.p. 175-177 °C (ref.[7] 174-176 °C), $[\alpha]_D^{20}$ = +52° (c = 0.55 $CHCl_3$). ^{1}H NMR: δ = 1.06 (d, 3 , *J* = 5.97 Hz, 6-Me), 1.15-1.29 (m, 1 , 1.50 (S, 3 H, 3-Me), 1.52-1.78 (m, 5 H), 1.83-1.91 (m, 1 H), 1.94-2.05 (m, 1 H), 2.11-2.18 (m, 1 H), 2.38-2.49 (m, 1 H), 2.60-2.66 (dd, 1 H, *J* = 4.6, 13.8 Hz), 6.28 (s, 1 H, H-12). - ^{13}C NMR: δ = 19.50, 24.07, 25.37, 25.86, 33.02, 35.53, 37.52, 50.01, 52.16, 81.82, 94.28, 105.97, 153.50, 187.89. MS (CI, CH_4): *m/z* (%) = 283 (44), 267 (100), 265 (56), 251 (34).

Method 2: Ruthenium(III) Chloride/Sodium Periodate

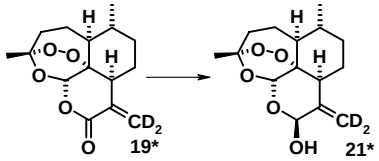
Artemisitene **19** (1.675 g, 5.94 mmol) and sodium periodate (5.246 g, 24.53 mmol, 4.1 equiv.) were dissolved in a mixture of carbon tetrachloride (32 mL), acetonitrile (32 mL) and water (48 mL), according to a general method.[8] The mixture was stirred vigorously whilst ruthenium(III) chloride hydrate (27.3 mg, 0.022 equiv) was slowly added. The mixture was stirred vigorously at room temperature for 1.5 h, until analysis of the reaction mixture by tlc indicated disappearance of the starting material. Dichloromethane (160 mL) was added, and the organic layer was separated. The aqueous layer was extracted with dichloromethane (3 × 100 mL). The combined organic extracts were dried ($MgSO_4$) and then filtered through a pad of silica gel. After evaporation of the solvent, the crude product was purified by recrystallization from dichloromethane/diethyl ether. Off-white to pale brown crystals of **20** (0.909 g, 54%) were obtained. Although these were not as pure as the product obtained by ozonolysis, the product was shown by ^{1}H NMR spectroscopy to be the same as the product from ozonolysis.

*Conversion of dicarbonyl compound **20** into 9-[C^2H_2]-artemisitene **19***:*



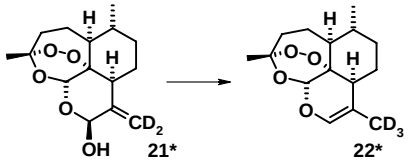
A solution of methyl-D_3 triphenylphosphonium bromide (Aldrich, 95+ atom % D, 766.4 mg, 2.128 mmol, 1.2 equiv) in THF (15 mL) under a nitrogen atmosphere was cooled to -78 °C, and treated dropwise with *n*-butyllithium (1.78M in hexanes, 1.2 mL, 2.14 mmol, 1.2 equiv.). During the addition, the colour of the solution become orange. The solution was stirred for 3 h, and then a solution of the dicarbonyl compound **20** (500 mg, 1.773 mmol) in THF (15 mL) was added at -78 °C. The resulting mixture was kept at -78 °C for 4 h and then slowly warmed to room temperature. The entire reaction time was 15 h. The reaction mixture was quenched with saturated aqueous ammonium chloride (15 mL), diluted with water (~10 mL) and then extracted with ethyl acetate (3 ×25 mL). The organic extracts were dried ($MgSO_4$) and then filtered. The filtrate was evaporated under reduced pressure to give the crude product as a yellow-brown solid. It was purified by chromatography with ethyl acetate-hexanes (20:80) to give a white crystalline solid of **19*** (215.5 mg, 43%), m.p. 166.4 - 167.7 °C. ^{1}H NMR: δ = 1.02 (3H, d, 6-Me, *J* = 5.7 Hz), 1.26-1.15 (1H, m), 1.46 (3H, s, 3-Me), 1.64-1.48 (4H, m), 1.81-1.72 (2H, m), 2.10-1.96 (2H, m), 2.46-2.36 (1H, m), 2.58-2.52 (1H, dd, *J* = 4.39, 4.53 Hz), 5.98 (1H, s, H-12). MS (CI, CH_4): *m/z* (%) = 283 (34), 265 (52), 237 (100), 219 (48), 179 (84). Exact mass: calcd, for $C_{15}H_{19}D_2O_5^+$ = 283.1513 found 283.1486.

Reduction of 9-[C^2H_2]-artemisitene to 9-[C^2H_2]-dihydroartemisitene **21***



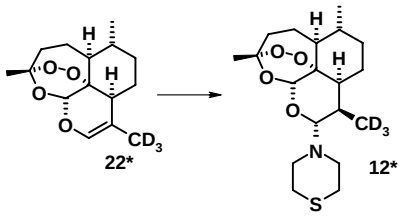
A stirred solution of 9-[C^2H_2]-artemisitene **19*** (400 mg, 1.42 mmol) in dichloromethane (15 mL) at -78 °C under nitrogen was treated dropwise with diisobutylaluminium hydride (1 M in dichloromethane, 2.7 mL, 1.9 equiv). The resulting mixture was stirred for a further 2.5 hours at -78 °C, and then quenched with aqueous sodium hydrogen sulfate (5 M, ~10 mL). It was diluted with water (~5 mL), and the dichloromethane layer was separated. The aqueous layer was extracted with dichloromethane (3 × 10 mL). The combined organic extracts were washed with water (15 mL), and then dried ($MgSO_4$). The solvent was evaporated to leave a white crystalline residue of 9-[C^2H_2]-dihydroartemisitene **21*** (337.5 mg, 84%), which was recrystallised from ethyl acetate to give needle shaped crystals, m.p. 164.2 - 166.4 °C. ^{1}H NMR: δ = 0.994-0.973 (3H, d, *J*= 6.3 Hz, H-6), 1.254-1.057 (1H, m), 1.257-1.352 (2H, m), 1.426 (3H, s, H-3), 1.753-1.625 (2H, m), 1.881-1.937 (2H, m), 2.118-2.027 (2H, m), 2.392-2.268 (2H, m), 2.835 (1H, s, OH), 5.739 (1H, s, H-12), 5.776-5.763 (1H, d, *J*= 3.9 Hz, H-10). Exact mass: calcd, for $C_{15}H_{20}D_2O_5$ = 284.1591, for $C_{15}H_{19}D_2O_5$ = 283.1513, found 283.1487.

Ionic reduction of 9-[C^2H_2]-dihydroartemisitene to 9-[C^2H_3]-glycal ***22****



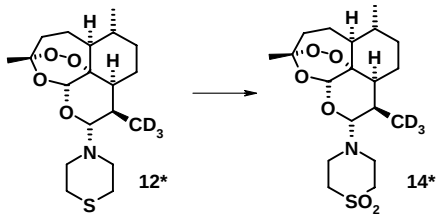
Triethylsilane-*d* (1.5 mL, 4.1 equiv) was added to a solution of the crude 15-CD_2-dihydroartemisitene **21*** (0.66 g, 2.31 mmol) in dichloromethane (33 mL) at -78 °C under nitrogen, and the resulting solution was stirred at -78 °C for 10-15 min. Trifluoroacetic acid (360 μL, 2 equiv) was then added, and the resulting mixture was stirred for 4 hours at -78 °C. It was then quenched with saturated aqueous ammonium chloride solution (17 mL). The dichloromethane layer was separated and the aqueous layer was extracted with dichloromethane (2 × 15 mL). The organic layers were combined, and washed with brine (15 mL), and then dried. After filtration, the filtrate was evaporated under reduced pressure to leave the crude product as an oil. This was immediately taken into ethyl acetate-hexane (10:90) and submitted to column chromatography over silica gel to give the 9-[C^2H_3]-glycal **22*** (252 mg, 40% yield). Recrystallisation from hexane gives needle shaped crystals, m.p. 91.4 – 92.3 °C. ^{1}H NMR: δ = 0.970-0.991 (d, *J* = 6.3 Hz, 3H, 6-Me), 1.077-1.252 (m, 3H), 1.424 (s, 3H, 3-Me), 1.515-1.733 (m, 3H), 1.894-2.173 (m, 4H), 2.349-2.441 (m, 1H), 5.528 (s, 1H, H-12), 6.170 (s, 1H, H-10). MS (CI, CH_4): *m/z* (%) = 270 (64), 269 (88), 252 (80), 224 (60), 206 (38), 176 (48), 166 (100). Exact mass: calcd. for $C_{15}H_{20}D_3O_4^+$ = 270.1782, found 270.1770.

Conversion of 9-[C^2H_3]-glycal into 9-[C^2H_3] thiomorpholine derivative **12***



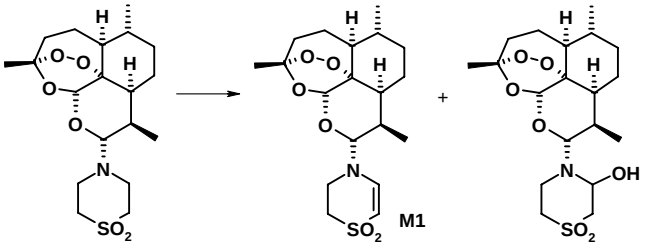
Hydrogen chloride gas was generated by dropwise addition of concentrated H_2SO_4 onto NH_4Cl powder at a rate of 1 drop per 5 s with a flow rate of *ca*. 5.5 mmol per min. The HCl gas was passed over a stirred mixture of 9-[C^2H_3]-glycal **22*** (100 mg, 0.372 mmol) in dichloromethane (6 mL) at 0 °C. After 10 min, the system was flushed with nitrogen for 10 min. Then, under an atmosphere of nitrogen, a mixture of thiomorpholine (0.043 mL) and triethylamine (0.054 mL) in dichloromethane (~1 mL) was added. After 45 min, the reaction mixture was quenched with brine (3 mL). The organic layer was separated and the aqueous layer was extracted with dichloromethane (2 × 2 mL). The combined organic extracts were dried ($MgSO_4$). Filtration and evaporation of filtrate gave a pale yellow solid, which was then purified by column chromatography with solvent ethyl acetate:hexane (8:92) to obtain the 9-[C^2H_3]-thiomorpholine derivative **12*** (77.5 mg, 56%). ^{1}H NMR: δ = 0.933-0.953 (d, *J* = 6.0 Hz, 3H, 6-Me), 0.933-1.053 (m, 1H), 1.207-1.704 (m, 8H), 1.392 (s, 3H, 3-Me), 1.833-1.900 (m, 1H), 1.961-2.024 (m, 1H), 2.286-2.393 (m, 1H), 2.571-3.265 (m, 8H), 3.929-3.963 (d, *J* = 10.2 Hz, 1H, H-10), 5.248 (s, 1H, H-12). MS (CI, CH_4): *m/z* (%) = 373 (100), 327 (80), 270 (12), 224 (10), 104 (16). Exact mass: calcd, for $C_{19}H_{29}D_3NO_4S^+$ = 373.2237, found 373.2222.

*Preparation of 9-[C^2H_3]-Artemisone **14****



m-Chloroperbenzoic acid (71%, standardized as described above) (71.0 mg, 0.290 mmol, 2.0 equiv) and potassium carbonate (202.8 mg, 1.5 mmol, ~10.0 equiv) was added into a stirred solution of the 9-[C^2H_3].thiomorpholine derivative **12*** (54.1 mg, 0.145 mmol) in diethyl ether (7 mL) at 0 °C. The reaction mixture was allowed to stir for 5 hours at 0 °C. The reaction mixture was treated with saturated sodium bicarbonate (15 mL), the organic layer was separated and it was washed with saturated sodium bicarbonate (15 mL). The aqueous layer was extracted once with diethyl ether (15 mL), and the combined organic extracts was then washed with brine (10 mL) and dried ($MgSO_4$). The solvent was evaporated under reduced pressure to leave a white solid (42.3 mg, 72% yield), which was shown by ^{1}H NMR spectroscopy to be essentially pure 9-[C^2H_3]-artemisone **14**. A small amount of sample was recrystallized from isopropanol to give needles, m.p. 147.7 – 148.3 °C. ^{1}H NMR: δ = 0.97-0.95 (3H, d, J = 6.3 Hz, 6 Me), 1.07-0.99 (1H, m), 1.35-1.20 (4H, m), 1.38 (3H, s, 3 Me), 1.52-1.43 (1H, m), 1.74-1.69 (2H, m), 1.92-1.83 (1H. m), 2.04-1.97 (1H, m), 2.40-2.30 (1H, m), 2.59-2.54 (1H, dd, J = 10.5 Hz, 4.8 Hz, H-9), 3.26-3.12 (4H, m), 3.53-3.33 (4H, m), 4.22-4.19 (1H, d, J = 10.2 Hz, H-10), 5.27 (1H, s, H-12). MS (CI, CH_4): *m/z* (%) = 405 (68), 359 (100), 345 (40), 270 (22), 224 (28). Exact mass: calcd, for $C_{19}H_{29}D_3NO_6S^+$ = 405.2136, found 405.2129.

3. Preparation of Metabolite M1



Ozone was generated by passage of oxygen through a $TRIO_3GEN$ Model TOG A2 ozonizer and was passed over a stirred cold (-78 °C) solution of artemisone (1 g, 2.494 mmol) in dichloromethane (25 mL) for 2.5 hours. The reaction mixture was then purged with nitrogen until the blue colour disappeared. It was then warmed up to warm temperature and the solvent was evaporated under reduced pressure. The products was isolated by column chromatography with ethyl acetate-hexanes (30:70) followed by (60:40) to give the hydroxyl product as a gum (74.0 mg, 5.5%, ca. 77% pure with inseparable starting material). ^{1}H NMR: δ = (Only selected peaks were identified) 0.81-0.83 (3H, d, J = 6.9 Hz, 9 Me), 0.96-0.98 (3H, d, J = 6.3 Hz, 6 Me), 1.39 (3H, s, 3 Me),

3.79-3.87 (1H, m), 4.03-4.05 (2H, m), 4.07-4.10 (2H, m), 5.44 (1H, s, H-12), 5.83-5.86 (1H, d, J = 10.8 Hz, H-12). ^{13}C NMR: δ = 12.77, 20.54, 21.82, 25.00, 26.19, 29.56, 34.31, 36.40, 37.35, 37.66, 45.49, 51.32, 51.66, 57.94, 79.39, 80.35, 91.94, 104.78, 161.85. MS (CI, CH_4): *m/z* (%) = 416 (12), 402 (100), 342 (40), 267 (98). Exact mass: calcd, for $C_{19}H_{32}NO_7S^+$ = 418.1899, found = 418.1643. The elimination product **M1** as a pale yellow foamy solid (167.9 mg, 16.9%), m.p. = 150.9-151.9 °C. $[\alpha]_D^{22}$ = +36.6 (*c* = 0.94, $CHCl_3$), ^{1}H NMR: δ = 0.81-0.83 (3H, d, J = 6.9 Hz, 9 Me), 0.93-0.98 (3H, d, J = 5.7 Hz, 6 Me), 1.40 (3H, s, 3 Me), 1.58-167 (1H, m), 1.71-1.77 (2H, m), 1.85-1.94 (1H, m), 1.99-2.10 (2H, m), 2.19-2.42 (5H, m), 2.56-2.63 (1H, m), 3.08-3.21 (2H, m, H at the thiomorpholine ring), 3.77-3.86 (1H, m, H at the thiomorpholine ring), 4.01-4.06 (1H, m, H at the thiomorpholine ring), 4.38-4.42 (1H, d, J = 10.5 Hz, H=10), 5.15-5.17 (1H, d, J = 8.7 Hz, vinyl H at the thiomorpholine ring), 5.39 (1H, s, H-12), 6.60-6.63 (1H, d, J = 8.7 Hz, vinyl H at the thiomorpholine ring). ^{13}C NMR: δ = 13.29, 20.53, 21.77, 25.01, 26.17, 30.32, 34.30, 36.42, 37.69, 45.41, 47.92, 51.60, 80.02, 90.87, 91.84, 96.58, 104.82, 142.04. IR (film): ν_{max} = 703, 738, 828, 881, 928, 944, 1038, 1109, 1172, 1226, 1266, 1378, 1458, 1611, 2350, 2873, 2928, 3056 cm^{-1}. MS (CI, CH_4): *m/z* (%) = 400 (32), 382 (16), 354 (76), 340 (52), 312 (10), 267 (42), 221 (100). Exact mass: calcd, for $C_{19}H_{30}NO_6S^+$ = 400.1794, found = 400.1734.

Unchanged artemisone (539.6 mg, 54%) was recovered.

4. Properties of Artemisone: Preparative and Structural Aspects of Artemisone: X-Ray Crystallography for Artemisone

Introduction

The synthetic route commences with artemisinin, which was first isolated by the Chinese from the herb *Artemisia annua*. Artemisinin is a crystalline compound, whose absolute configuration has been established by X-ray diffraction employing Cu-*K*α radiation, anomalous dispersion, and by examination of Cotton effect.[9,10] The structure is as depicted in Figure 1. Artemisinin is a sesquiterpene lactone which may be classified under the amorphane sub-group of cadinane sesquiterpenes.[11] It is enantiomerically pure, and is biosynthesized according to the isoprene rule. It is a structurally unique natural product in possessing the 1,2,4-trioxane unit, that is, a peroxide bridge consisting of two contiguous oxygen atoms linked to a carbon atom which itself is linked to a third oxygen atom. Artemisinin crystallizes readily.[12,13] It has been converted into a very large number of derivatives, and all data are consistent with the absolute configuration of the parent compound designated in Figure 1.[11,14]

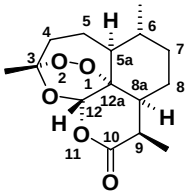


Figure 1: Structure of artemisinin

The Chemical Abstracts index name is based on its absolute configuration, where the chiral (chirogenic) centres are defined as (*R*)- or (*S*)- according to the Cahn-Ingold-Prelog system of designating absolute stereochemistry. This is (3*R*,5a*S*,6*R*,8a*S*,9*R*,12*S*,-12a*R*)-octahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4,3-j]-1,2-benzodioxepin-10(3*H*)-one, with Chemical Abstracts Registry Number (RN) 63968-64-9.

Artemisinin is reduced with sodium borohydride in methanol to the lactol dihydroartemisinin (DHA) (Figure 2).[10] Dihydroartemisinin is demonstrated to exist *in solution* as an equilibrating *mixture* of epimers (isomers) with so-called 10α-hydroxyl and 10β-hydroxyl groups.[15] The configuration of the epimers only differs at C-10, that is, at the epimeric (anomeric) centre of the lactol. The ratio of the 10α:10β epimers in chloroform is about 1:1, in acetone 2:1, and in dimethyl sulfoxide, is about 3:1, as determined by ^{1}H NMR spectroscopy.[16,17,18] The solid substance is believed to be the 10β-epimer, based on an X-ray crystallographic determination of a single crystal.[16,19] The CA RN for the β-epimer is 71939-50-9, and the index name is (3*R*,5a*S*,6*R*,8a*S*,9*R*,-10*S*,12*R*,12a*R*)-decahydro-3,6,9-trimethyl-3,12-epoxy-12*H*-pyrano[4,3-j]-1,2-benzodiox-epin-10-ol, consistent with the structural designation in Figure 2.

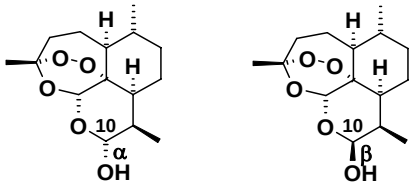


Figure 2: Dihydroartemisinin epimers

DHA has been converted by the Chinese into artemether and artesunate, and the structures of these pure, stable substances have been thoroughly established by means of ^{1}H NMR spectroscopy and X-ray crystallography.[16,17] Artemether is the β-epimer and artesunate is the α-epimer (Figure 3); the reasons for the formation of these configurationally pure compound from the mixture of DHA epimers in solution are easily understood.[17] A large number of other derivatives has been prepared from dihydroartemisinin, and here also, structures in some cases have been confirmed by X-ray crystallography and NMR analysis.[11,14,17]

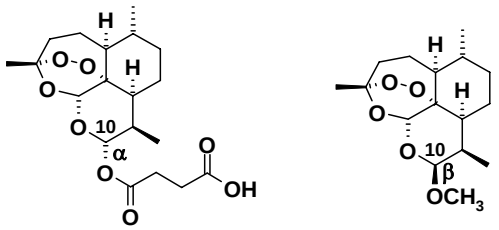


Figure 3: Structures of artesunate and artemether

Preparation of Artemisone

Artemisone is prepared in two steps (route a, Figure 4) or in one step (route b, Figure 4) from DHA. In route a, DHA is treated with NaBr and trimethylsilyl chloride in dichloromethane, under which conditions it is converted *in situ* in a novel process into the DHA 10-β bromide. This is then treated with thiomorpholine in the same solvent to give the DHA thiomorpholine derivative (compound **12**) in yields ranging between 54-77%. This is then oxidized with *N*-methylmorholine-*N*-oxide and a catalytic amount of the Ley reagent tetrapropylammonium perruthenate in quantitative yield. In the route b, thiomorpholine *S*,*S*-dioxide is added to the 10-β-bromide generated *in situ* to give artemisone directly, but in a lower yield (44%). The latter direct route is currently under optimization, and several other reagents have been identified which will improve the yield for this step; IP protection is required before details can be released.

The stereochemistry at C-10 is established in all conversions by means of ^{1}H NMR spectroscopy.

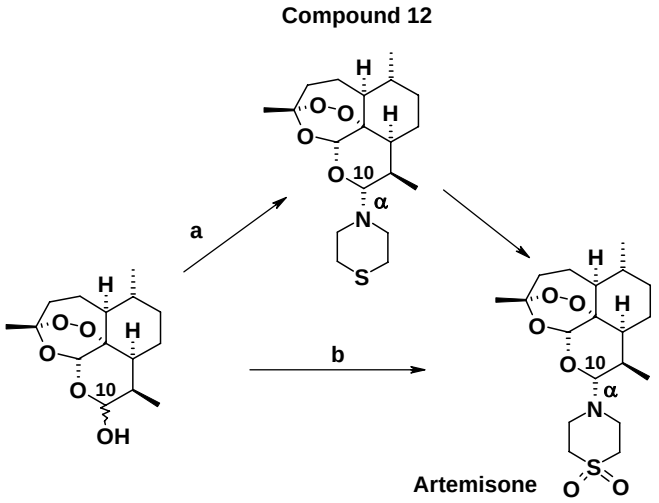


Figure 4: Preparation of artemisone from dihydroartemisinin.

Structural Features.

It is established that artemisone is produced as a single compound. It crystallizes as monoclinic crystals, irrespective of the solvent of crystallization. Larger scale preparation (to 20 gram scale at HKUST; to 20 kg pilot process scale according to routes a or b at Bayer) provides only a single compound; this is verified by examination of successive crops obtained by successive crystallizations of mother liquors, and examination of the crystals obtained by ^{1}H NMR spectroscopy. Both the structures, including absolute configurations, of the precursor of artemisone, namely BAY 44 9584 and of artemisone, have been established by X-ray crystallography (see below). This is possible because each compound contains a heavy atom (sulfur), and the anomalous dispersion from the heavy atom unambiguously provides the absolute configuration. As DHA is derived from artemisinin, the configuration of the chiral (chirogenic) centres at the carbon atoms in DHA (with the exception of C-10), are identical to those of artemisinin. Similarly, the conversion of DHA into artemisone does not affect the configuration of the carbon atoms, and this is also verified by the X-ray crystallography.

The ^{1}H NMR spectroscopic data is also consistent. The signal due to H-10 in artemisone appears as a doublet at δ 4.38 ppm, with coupling constant (J = 10.3 Hz) (Figure 5). This is *only* consistent with a diaxial coupling with H-9;[16,17,19] therefore both H-10 and H-9 are in a trans-diaxial arrangement in a chair pyran ring (Figure 6). Therefore, the thiomorpholine *S*,*S*-dioxide ring is in the α-configuration, and must be attached by an equatorial bond. The compound is configurationally stable, and no *trace* of any compound containing the thiomorpholine *S*,*S*-dioxide ring attached in the β-configuration is ever detected. The X-ray structure also indicates that the thiomorpholine *S*,*S*-dioxide ring is attached to C-10 by an equatorial bond, and the pyran ring incorporating C-10 is in the chair configuration.

The geometry is also apparent from a molecular model of artemisone, minimized using semi-empirical calculation (AM1) on Spartan '02 for Windows (Figure 7). Here the chair configuration of the pyranose ring, the trans-diaxial arrangement of H-9 and H-10, and the equatorial attachment of the thiomorpholine *S*,*S*-dioxide ring are clearly apparent.

The Chemical Abstracts name for artemisone based on these structural determinations is 4-[(3*R*,5a*S*,6*R*,8a*S*,9*R*,10*R*,12*R*,12a*R*)-decahydro-3,6,9-trimethyl-3,12-epoxy-12*H*-pyrano[4,3-j]-1,2-benzodioxepin-10-yl]thiomorpholine 1,1-dioxide, RN 255730-18-8.

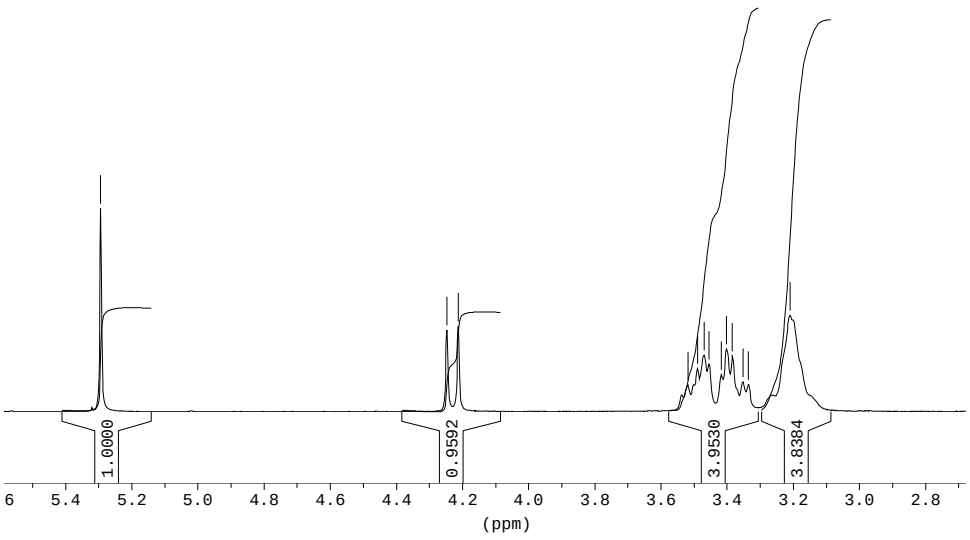


Fig. 5: 300 MHz ^{1}H NMR spectrum of artemisone in $CDCl_3$; expansion in the region 2.8-5.5 ppm and analysis of the signal due to H-10 as a doublet, J = 10.3 Hz centered at 4.38 ppm.

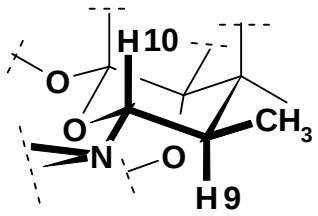


Figure 6: Part structure of artemisone indicating trans-diaxial relationship of H-9 and H-10 in the pyran ring.

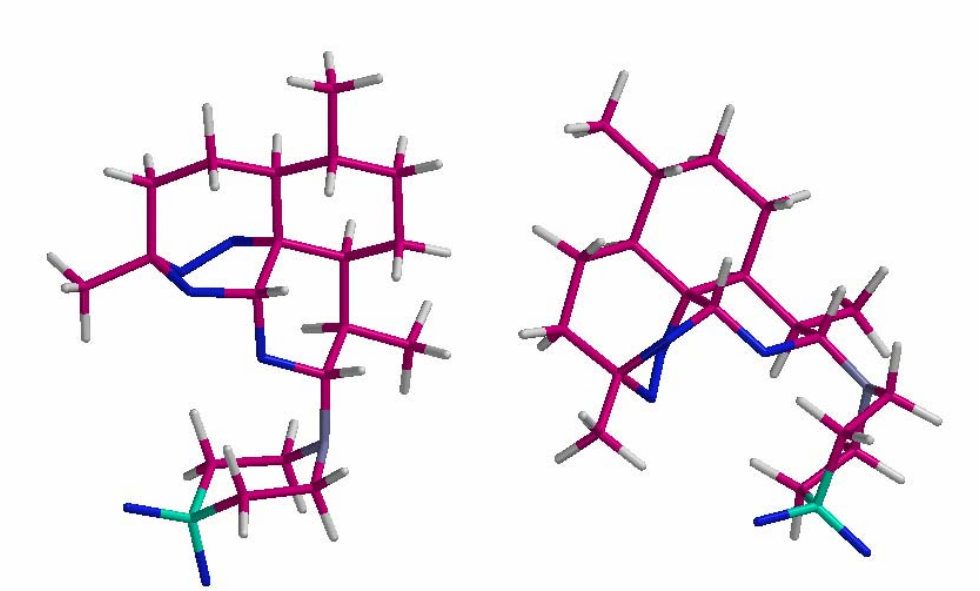


Figure 7: Two views of AM1 geometry-optimized structure of artemisone indicating trans-diaxial relationship of H-9 and H-10 in chair pyran ring, and the equatorial bond at C-10 attached to the thiomorpholine *S*,*S*-dioxide ring

Crystal Structure Determination

The compound artemisone is an artemisinin derivative of empirical formula [$C_{19}H_{31}NO_6S$] with a pendant 1-aza-4-sulphona-cyclohexane ring. The compound crystallizes in the monoclinic system with cell parameters a = 11.2017(7), b = 12.5252(7), c = 15.2893(9)Å, β = 108.564(1)^o, V = 2046.8(2)Å^3. The systematic absences 0k0 = 2n+1 were indicative of chiral space group $P2_1$, consistent with enantiomeric purity. A suitable crystal of dimensions 0.5 x 0.4 x 0.3 mm^3 was mounted on a glass fiber and intensity data collected at room temperature on a Bruker SMART diffractometer, equipped with a CCD unit. A total of 8614 unique intensity data ($2\Theta_{max}$ = 56^o) were used to solve and refine the structure using 487 parameters. All non-hydrogen atoms were refined using anisotropic thermal parameters. All hydrogens were located in difference Fourier maps but then placed in geometrically constrained positions with isotropic thermal parameters derived from the carbon to which they were attached. Refinement proceeded smoothly and the final conventional R_1-value = 0.0459 (for I>2σ) and wR2 = 0.1160 (all data). The Flack parameter 0.25(7) was consistent with the expected absolute stereochemistry, though the low anomalous dispersion meant this had a high standard deviation. All computations were carried out using the SHELX suite of X-

ray programs.[20] A summary of the crystal data and refinement details are given in Table 1.

Table 1: Crystal Data and Structure Determination Summary for Artemisone

Empirical formula	$C_{19}H_{31}NO_6S$	Volume	2046.8(2) $Å^3$
Formula weight	401.51	Z	4
Temperature	293(2) K	Density (calculated)	1.303 Mg/m^3
Wavelength	0.71073 A	Absorption coefficient	0.192 mm^{-1}
Crystal system	Monoclinic	F(000)	864
Space group	P2(1)	Crystal size	0.5 x 0.4 x 0.3 mm
Unit cell dimensions	a = 11.2017(7) Å	Theta range	1.40 to 28.03
	b = 12.5252(7) Å	Limiting indices	-14<=h<=14,
	c = 15.3893(9) Å		-16<=k<=15,
	β = 108.564(1) ^{o}		-20<=l<=18
Volume	2046.8(2) $Å^3$	Final R indices [I>2σ(I)]	R1 = 0.0459,
Z	4		wR2 = 0.0925
Density (calculated)	1.303 Mg/m^3	R indices (all data)	R1 = 0.1028,
Independent reflections	8630 [R(int) =		wR2 = 0.1160
0.0325]		Absolute structure	0.25(7)
Refinement method	Full-matrix least-squares on F^2	parameter (Flack)	
Data / restraints / parameters	8614 / 1 / 487	Largest diff. peak/ hole	0.167 and -0.214
Goodness-of-fit on F^2	0.932	e.$Å^{-3}$	

The unit cell has four molecules, so that there are two independent molecules per asymmetric unit, which are designated A and B. The stereochemistry and geometric parameters for the two molecules are similar. A thermal ellipsoid plot of molecule A is shown in Fig. 8. The aza- functionalities N(1A) and N(1B) are unprotonated and exist as tertiary amine free-bases in the crystal. This is supported by their pyramidal geometry, the absence of significant residual electron density (+0.17e$Å^{-3}$) in the final difference map and also by the lack of any hydrogen bond contacts for these nitrogen atoms. The sulfone groups have S=O bond lengths in the range 1.427-1.52(3)Å, consistent with double bond character. The pendant rings adopt chair conformations with N atoms bent up to the artemisinin moiety and the SO_2 groups bent down and away. The peroxide functionalities are similar in the two independent molecules, the O-O distances are 1.462 and 1.467(3)Å. Both show the distinct asymmetry in bonding to the seven membered ring, with the C-O bond to the central carbon considerably longer (1.459(3), 1.472(3)Å) compared to the hemiacetal carbon (1.420, 1.415(3)Å). This feature is consistent with that seen in other artemisinin derivatives.

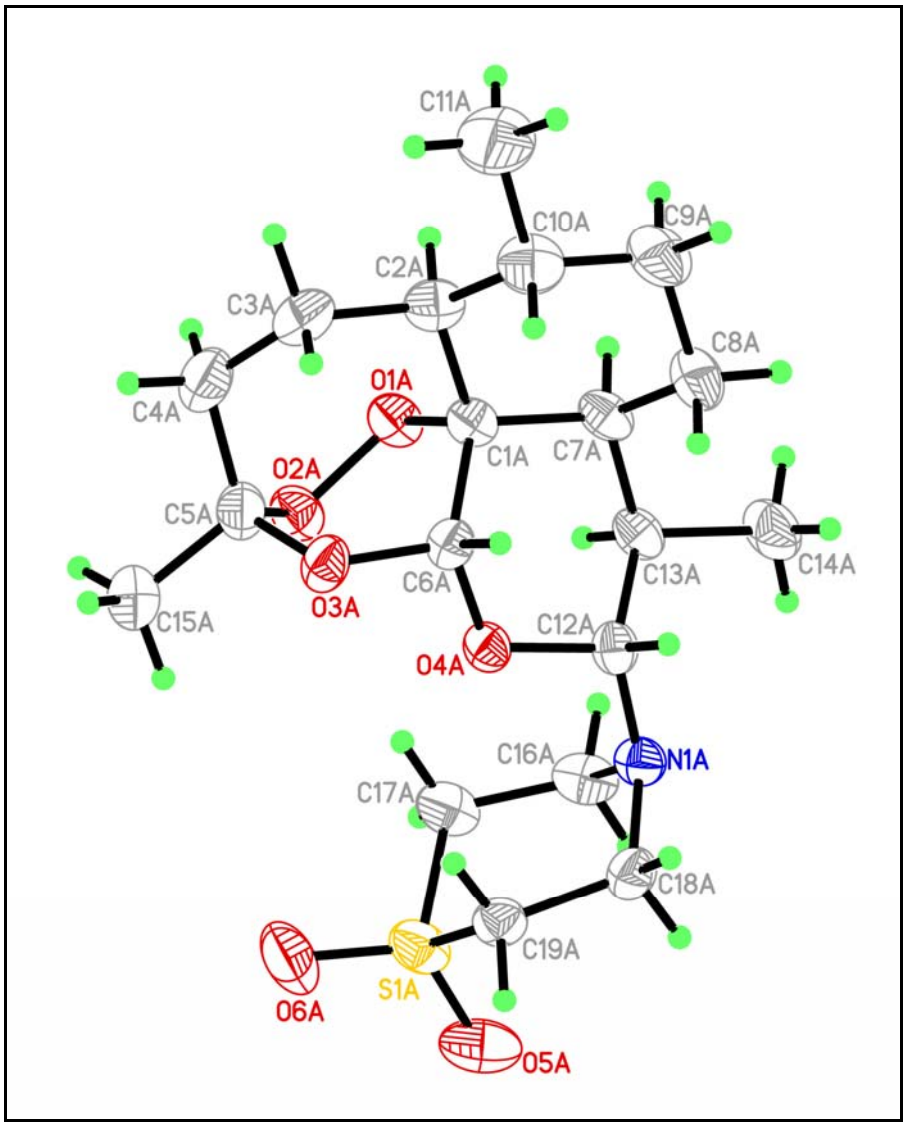


Figure 8: Thermal ellipsoid plot of artemisone with crystallographic numbering.

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[15] Each epimer of DHA has a chair pyranose ring. The 'α'-epimer has an equatorial hydroxyl, the 'β'-epimer an axial hydroxyl. However, this 'convention' which has grown from the 'normal' manner of representing structures of dihydroartemisinin derivatives in the artemisinin literature is the reverse of that normally used for designating stereochemistry of sugars and their glycosides, in which, for example, α-D-glucopyranose possesses an axial hydroxyl. This inconsistency is unfortunate, but for convenience, the artemisinin stereochemical 'convention' is used here.

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Artemisone - A New, Highly Active Antimalarial Drug of the Artemisinin Class

Richard K. Haynes, Burkhard Fugmann, Karl Rieckmann, Hans-Dietrich Heilmann, Ho-Wai Chan, Man-Ki Cheung, Wai-Lun Lam, Ho-Ning Wong, Simon L. Croft, Livia Vivas, Lauren Rattray, Lindsay Stewart, Wallace Peters, Brian L. Robinson, Michael D. Edstein, Barbara Kotecka, Dennis E. Kyle, Bernhard Beckermann, Michael Gerisch, Martin Radtke, Gabriele Schmuck, Wolfram Steinke, Ute Wollborn, Karl Schmeer, Axel Römer

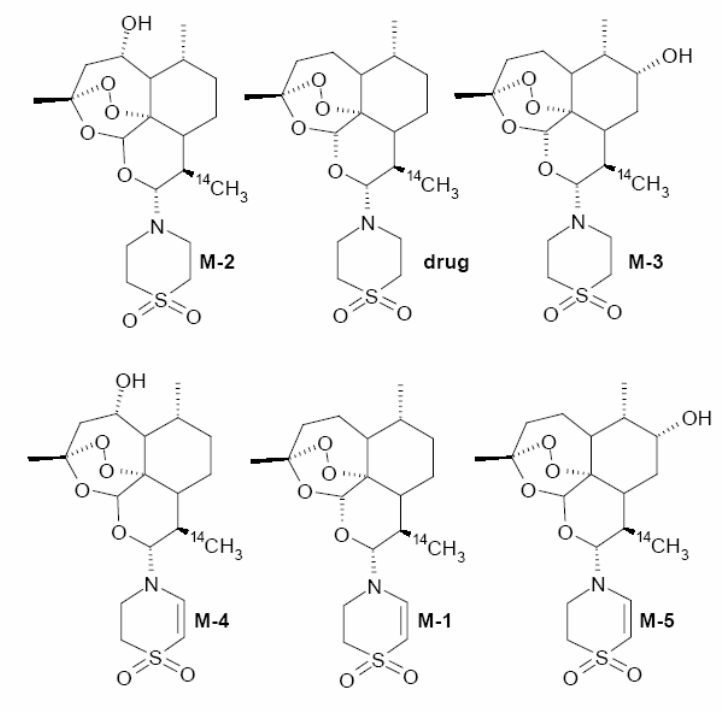
Supporting Information 2: Metabolites, Incubation Studies

1. Metabolites

[^{14}C]-Artemisone: Isolation and Structure Elucidation of Metabolites from Incubations with Human Liver Microsomes.

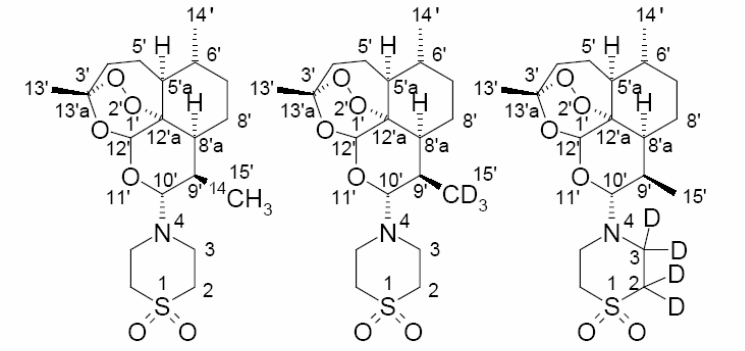
a. Summary

[14C]-Artemisone has been incubated with human liver microsomes to produce metabolites for (i) structure elucidation by 1H NMR spectroscopy and mass spectrometry, (ii) the chromatographic comparison with *in vitro* (hepatocyte incubations) and *in vivo* (plasma, bile, liver from rat/dog) samples and (iii) assessment of pharmacological efficacy. This report describes isolation and structure elucidation of five phase I metabolites which are expected to play the central role in metabolism of artemisone in man. The metabolites identified are: **M-1**, bearing a double bond in the thiomorpholine dioxide ring, **M-2** and **M-3**, hydroxylated in different positions of the tricyclic ring system, and **M-4** and **M-5**, formed by combination of the primary biotransformation reactions. The structures are depicted below:



b. Introduction

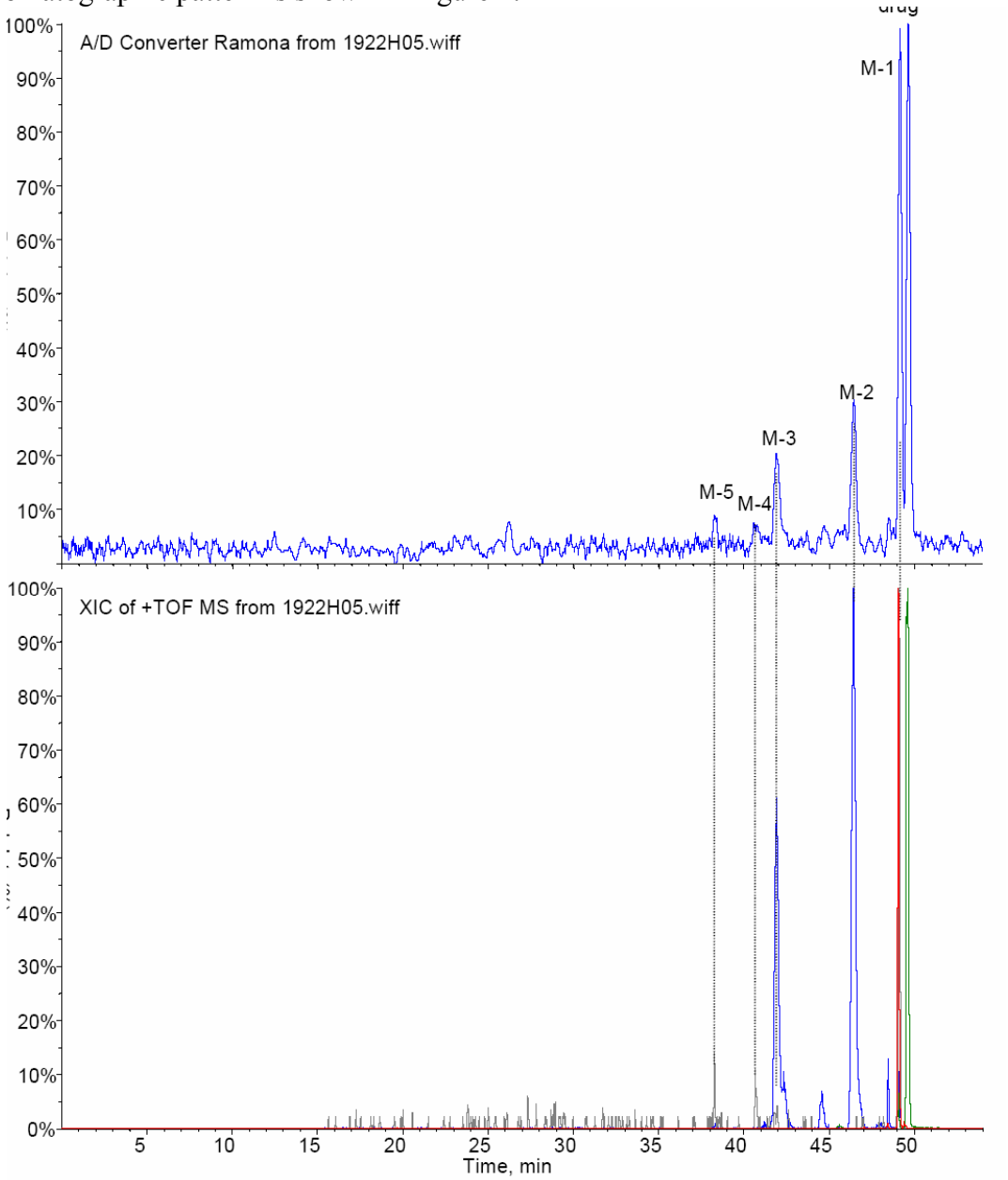
[14C]-Artemisone, 4-[(3'R, 5'aS, 6'R, 8'aS, 9'R, 10'R, 12'R, 12'aR)-3', 6', 9[14C]-trimethyldecahydro-3', 12'-epoxy[1', 2'] dioxepino[4', 3'-i]isochromen-10'-yl]thiomorpholine 1,1-dioxide1, is rapidly metabolized to several major metabolites in human and animal liver microsomal incubations *in vitro*. In order to fully elucidate the metabolite structures, the compound has been incubated with liver microsomes from man (mix) on a larger scale and the metabolites formed were isolated and purified. The isolated fractions were analyzed by HPLC- ^{14}C-UV-MS/MS and by ^{1}H- and 2D-NMR spectroscopy. The absolute configuration of the drug is given below and the shown numbering system for the hydroxy and dehydro-derivatives of artemisone is used.



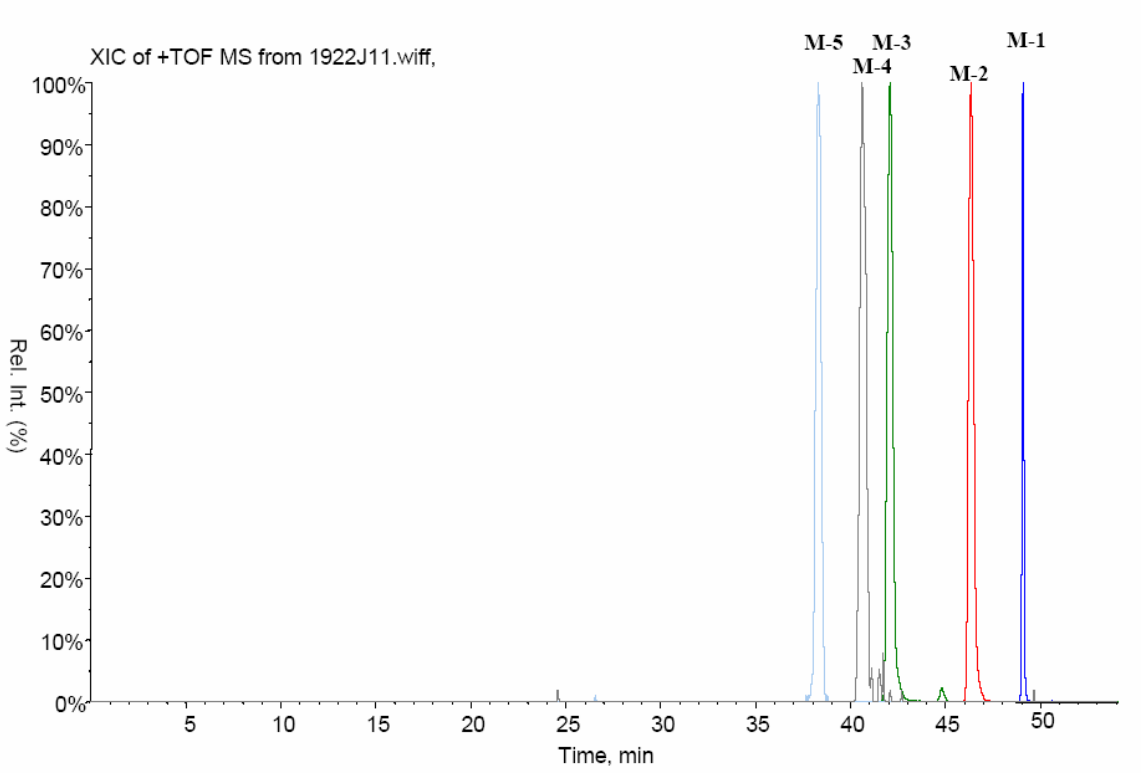
c. Results

c.1 Metabolites Isolated from Human Liver Microsomal Incubation

[14C]-Artemisone has been incubated with human liver microsomes(2). The chromatographic pattern is shown in Figure 1.



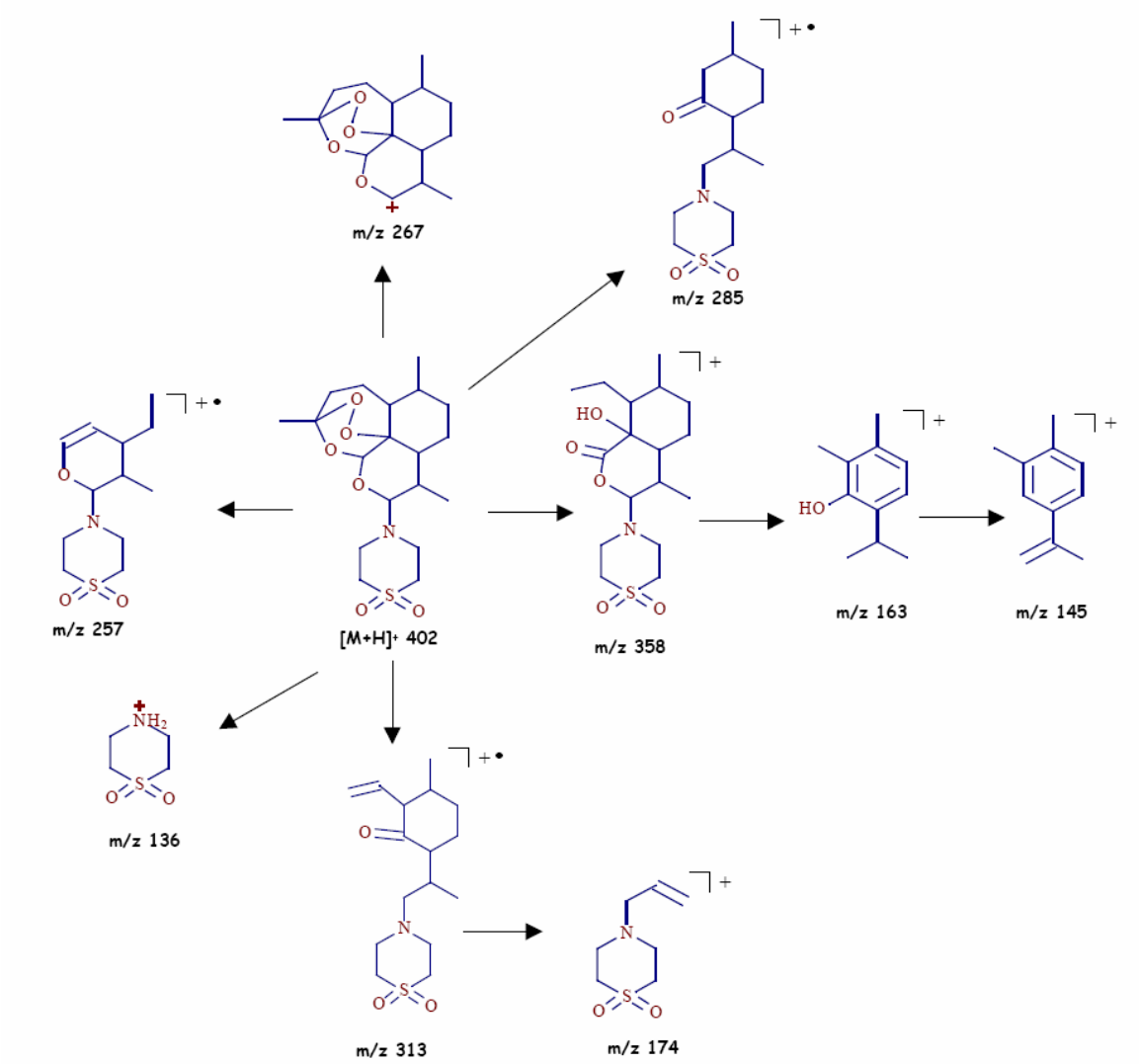
The extract of the reaction mixture was purified as described below and the metabolites were isolated by preparative HPLC. The resulting fractions were analyzed for purity by LC-^{14}C-UV-MS and were then subjected to ^{1}H NMR, 2D-NMRspectroscopy and mass spectrometry. The chromatographic pattern and the overlaid extracted ion currents (XIC's) are shown in Figure 1 and the ion chromatograms of the isolated fractions are shown in Figure 2. The incubations and work-up were performed as previously reported (2) and the following work-up is described in section 8.



c.2 Structure Elucidation by Mass Spectrometry

For the interpretation of mass spectra the unchanged drug, the ^{14}C-labeled, the ^{2}H3-labeled and the ^{2}H4-labeled drug have been subjected to an extensive mass spectrometric analysis by MS/MS and by MS/MS/MS. The product ions formed are depicted in the proposed mass spectrometric fragmentation pathway of artemisone (see below). The determination of the accurate mass by re-calibrating the TOF spectrum provided already - by the elemental composition - information on the kind of biotransformation, being hydroxylation, dehydrogenation and combinations of both. Hints on the site of the modification were collected from MS/MS-spectra. In order to obtain a maximum of fragments, different collision energies were employed (data stored with the file). An overview of the 12C fragmentation pathway is shown below. The key fragments were the ones at m/z 136, being the thiomorpholine-dioxide ring, and the corresponding artemisinyl-rest at m/z 267. These two, as well as the other fragments shown below, served mainly for the structure proposals based on MS data. The dehydrogenated thiomorpholine-dioxide ring with m/z 134 was the marker for **M-1** and the related structures of **M-4** and **M-5**. For the structure proposal of **M-2**, the intact fragment of 136 amu, (the thiomorpholine-dioxide ring) in combination with the fragment of 285 amu made C-4', C-5' and C-13' the tentative sites of hydroxylation. Further fragments made the ethylene bridge of C-4'and C-5' the most probable part. For the structure proposal of **M-3**, primarily the fragments with 301 amu, (being a 16 amu shift of the 285 amu fragment), together with the one at m/z 136, being the thiomorpholine dioxide ring, were taken into consideration. The spectra of **M-4** and **M-5** exhibited the characteristic signals from **M-1**/**M-2** and **M-1**/**M-3** which fitted well the structure proposals given by NMR.

Proposed mass spectrometric fragmentation pathway of artemisone (12C-compound)



c.3 Structure Elucidation by NMR

For the metabolite **M-1** all signals in the ^{1}H NMR spectrum remained at their position compared to the reference drug artemisone, except for the protons of the thiomorpholinedioxide ring. The integrals of the signals have changed from eight to four protons and two new signals at 5.22 and 6.65 ppm have been detected. These chemical shifts are characteristic for a double bond. The integrals correspond to two protons. In the COSY spectrum, a correlation signal between the two double bound protons could be detected. This is a clear sign that in the metabolite **M-1**, a double bond in the thio-morpholine-dioxide ring was incorporated. For the metabolite **M-2** most of the signals in the ^{1}H NMR spectrum remained at their position compared to the reference drug artemisone. A major shift was detected for one of the protons at C5' which have shifted from 1.45 and 1.88 ppm to 3.98 ppm, from two protons to one proton. For the two metabolites **M-2** and **M-3** it was possible to record the following heteronuclear two-

dimensional NMR experiments: 1H-13C-HSQC and 1H-13C-HMBC. By evaluation of the heteronuclear 2D-NMR experiments it was possible to assign the 13C chemical shifts of the metabolites **M-2** and **M-3**. The NMR data are summarized in Table 1 and Table 2. The 13C chemical shift of C5' changed from 24.8 ppm to 69.5 ppm. The changes of the chemical shifts from the proton and the carbon of C5' clearly indicated the oxidation of the methylene group to a hydroxymethine group. Additionally, a change of the chemical shifts of the protons and carbons at C4', C5a' and C6' to lower field indicated the electronegative influence of a hydroxy group at C5'. The stereochemistry of the hydroxy group at C5' was evaluated by a 1H-1H-NOESY spectrum. The NOE signals detected form the proton bound to C5' in **M-2** to the proton bound to C6' and the proton bound to C12' indicated the stereochemical configuration of the hydroxy group. For the metabolite **M-3** most of the signals in the 1H NMR spectrum remained at their position compared to the reference drug artemisone. A major shift was detected for one of the protons at C7' which has shifted from 1.04 and 1.70 ppm to 3.86 ppm, from two protons to one proton. The 13C chemical shift of C7' changed from 34.2 ppm to 69.8 ppm. The changes of the chemical shifts from the proton and the carbon of C7' clearly indicated the oxidation of the methylene group to a hydroxymethine group. Additionally, a change of the chemical shifts of the protons and carbons at C5a', C6', C8' and C8a' indicated the electronegative influence of a hydroxy group at C7'. The stereochemistry of the hydroxy group at C7' was evaluated by a 1H-1H-NOESY spectrum. The NOE signals detected form the proton bound to C7' in **M-3** to the protons bound to C8', C14 and not to the proton bound to C5a' indicated the stereochemical configuration of the hydroxy group. The metabolites **M-4** and **M-5** showed 1H-NMR spectra which were very similar to the 1H NMR spectra of **M-2** and **M-3**, respectively. The only difference occurred in the signals of the thiomorpholine-dioxide ring which were distracted and showed differences similar to the signals of **M-1**. Unfortunately, there was less material available for **M-4** and **M-5** and overlap with artefacts and may be distribution products are possible. It was not possible to extract the exact chemical shift of the probable double bound at the thiomorpholine-dioxide ring. This information, however could be supplied from mass spectrometry.

c 4 Tables

Table 1: 1H-Chemical shifts (ppm) of artemisone (BAY 44 9585) and the investigated metabolites. The protons are named by the C-atoms.

C-Atoms	BAY 44-9585	M-1	M-2	M-3	M-4	M-5
C2	3.21-3.26	3.18 / 5.22	3.15 / 3.28	3.15 / 3.28	3.0 / 3.28 / 3.40 / 3.72 / 3.85	3.32 / 3.90 / 4.08 /
C3	3.33-3.54	3.88 / 4.09 / 6.55	3.38 / 3.52	3.42 / 3.52		
C3'	-	-	-	-	-	-
C4'	2.00 / 2.35	2.07 / 2.40	2.55 / 2.55	2.06 / 2.41	2.51	2.06 / 2.45
C5'	1.45 / 1.88	1.52 / 1.94	3.98	1.50 / 1.84	3.98	1.52 / 1.84
C5'a	1.28	1.28	~1.34	1.84	1.38	1.84
C6'	1.35	1.37	1.55	1.55	1.55	1.55
C7'	1.04 / 1.70	1.07 / 1.78	1.12 / 1.76	3.86	1.15 / 1.80	3.88
C8'	1.35 / 1.70	1.35 / 1.78	1.38 / 1.76	1.56 / 1.94	1.37 / 1.80	~1.60 / 1.95
C8'a	1.60	1.67	1.67	2.17	1.69	2.25
C9'	2.59	2.64	2.64	2.66	2.73	2.68
C10'	4.22	4.42	4.28	4.22	4.40	4.38
C12'	5.29	5.42	5.28	5.31	5.30	~5.40 ?
C12'a	-	-	-	-	-	-
C13'	1.37	1.45	1.42	1.42	1.42	1.44
C14'	0.96	1.05	1.18	1.08	1.19	1.09
C15'	0.80	0.86	0.83	0.83	0.99	0.87

Table 2: 13C-Chemical shifts (ppm) of artemisone and the investigated metabolites **M-2** and **M-3**.

C-Atoms	BAY 44-9585	M-2	M-3
C2	51.9	50.8	51.9
C3	47.0	46.8	47.0
C3'	104.3	102.5	104.3
C4'	36.2	46.2	36.3
C5'	24.8	69.5	24.8
C5'a	51.5	58.6	43.9
C6'	37.5	37.0	41.1
C7'	34.2	34.9	69.8
C8'	21.6	21.6	28.8
C8'a	45.6	45.4	38.1
C9'	29.1	29.0	28.5
C10'	92.1	91.6	92.1
C12'	91.0	91.0	90.5
C12'a	80.2	79.5	79.8
C13'	25.9	25.5	25.8
C14'	20.2	21.3	16.3
C15'	13.4	13.4	13.2

c.5. Figures

Figure 1: Artemisone
The chromatographic pattern from incubations with human liver microsomes as 14C-trace (top) and XIC's (bottom).

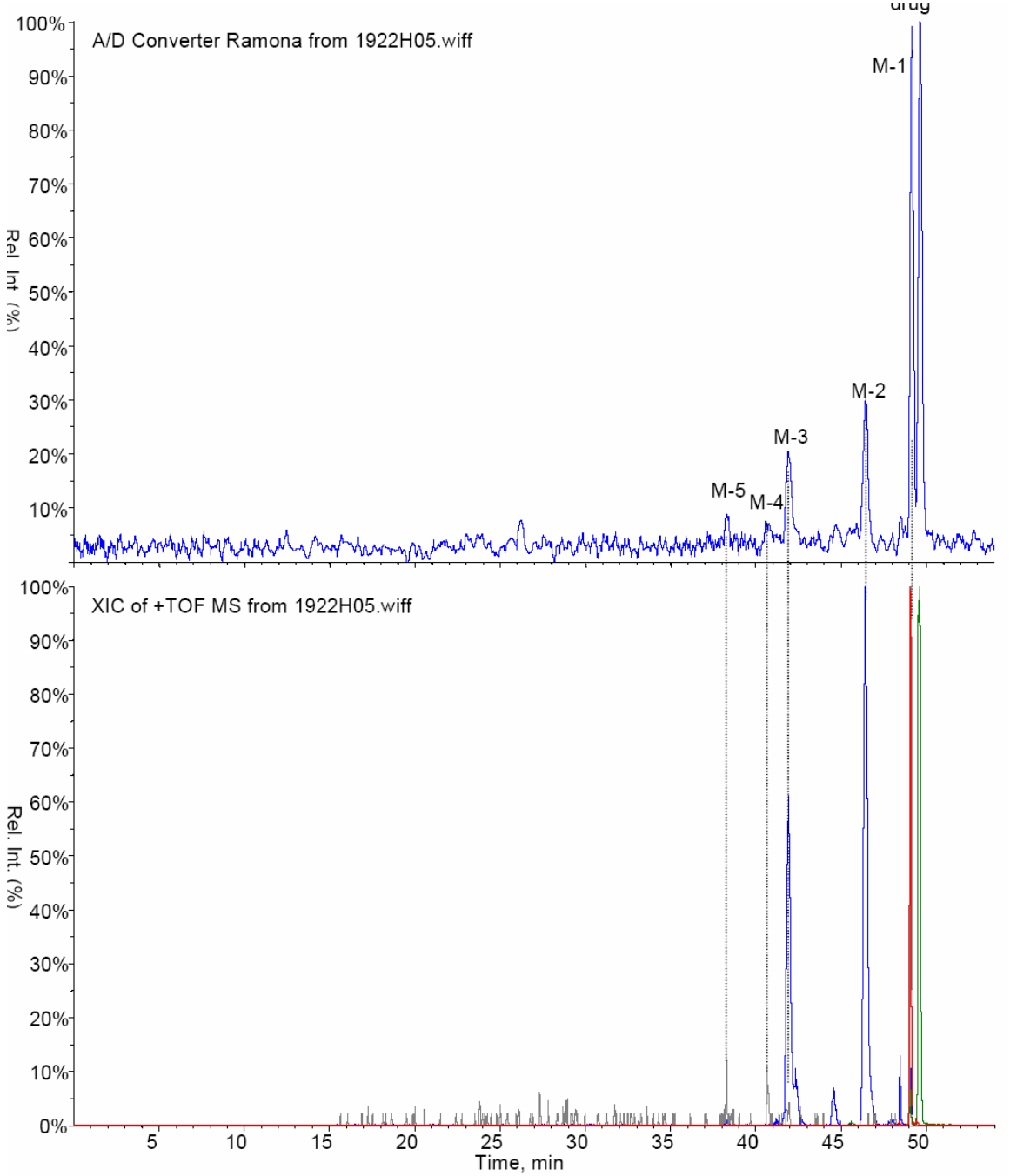


Figure 2: Artemisone
HPLC (XIC's) comparison of the five fractions -containing metabolites **M-1** to **M-5** - obtained from incubations with human liver microsomes.

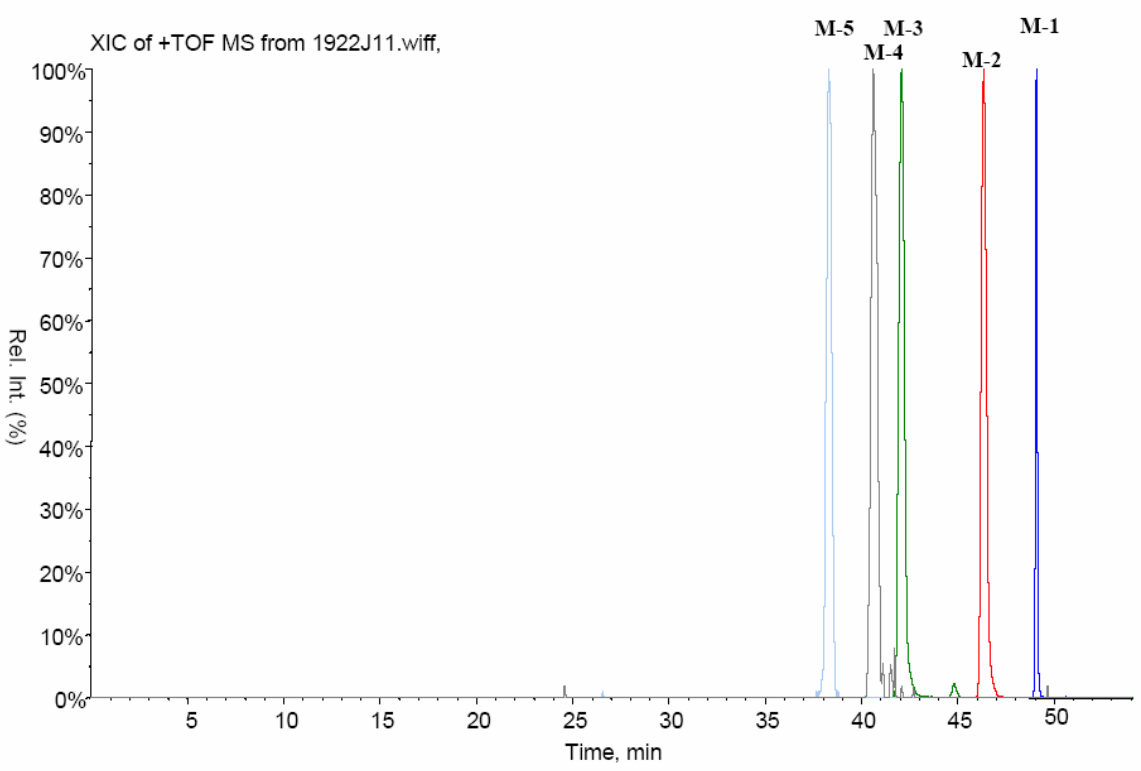
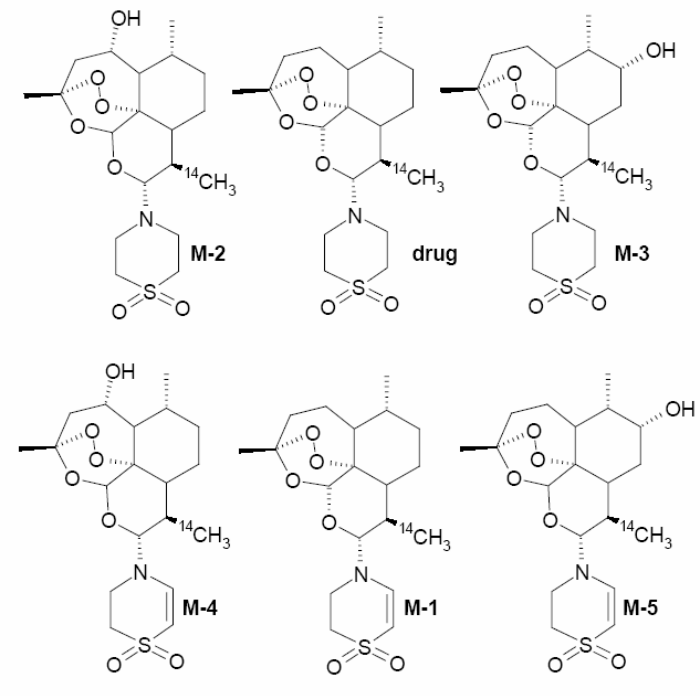


Figure 3: Artemisone
Metabolites identified in an incubation with human liver microsomes.



c. 6. Material and Methods

c. 6.1 Drug and Labeled Compound

The 14C-labeled drug, batch no. : GCM1330-1-01E specific activity : 2.42 MBq/mg,

was synthesized by Dr. M. Gerisch, PH-PD P MIC, Bayer HealthCare AG, Wuppertal, FRG1.

c. 6.2 Reagents
Chemicals
di-Potassium hydrogen phosphate no. 5101; magnesiumchloride hexahydrate for analysis no. 5833; saccharose no. 7653; raffinose pentahydrate no. 1.07419 (E. Merck, Darmstadt, FRG); acarbose (GT-Ware 1083/95HF1-3, Lab. Dr. Lenz, Bayer AG, Wuppertal, FRG). □-Cyclodextrine no. 28705 (Fluka, Buchs, Switzerland); formic acid 98 - 100 % R.G. no. 33015; ammonium acetate R.G. no. 32301; water Chromasolv® no. 34877; acetonitrile Chromasolv® no. 34851 (Riedel de Haen, Seelze, FRG); EDTA no. 11278; NADP no. 49207744; glucose 6-phosphate dehydrogenase no. 85913432; (Roche Diagnostics GmbH, Mannhein, FRG); glucose 6-phosphate no. 92338429 (Roche Diagnostics GmbH, Mannhein, FRG); Ultima Gold® high flash point scintillation cocktail (Packard Instrument BV Chemical Operations, Groningen, The Netherlands).

c. 6.3 Reagents and Equipment
Labofuge® A (Heraeus Christ, Osterode, FRG).
HPLC system, HP 1090 with diode array detector and work station (Hewlett-Packard, Waldbronn, FRG).
Radio-HPLC monitor: Ramona® 5 (Raytest GmbH, Straubenhardt, FRG).
Column oven: Mistral Slave® (Spark, Emmen, Netherlands),
with 6-ways-tandem-microvalve (SunChrom, Friedrichsdorf, FRG).
A/D-converter HP 35900 C (Hewlett-Packard, Waldbronn, FRG).
Radioactivity monitor Ramona® 2000 connected to HP-Chemstation
(Raytest, GmbH, Straubenhardt, FRG).
Capillary LC-System 1100 (Agilent, Waldbronn, FRG).
Q-Star-Pulsar® mass spectrometer with API source (Sciex, Toronto, Canada).
Liquid Scintillation spectrometer Tri-Carb® 2500 TR (Canberra, Packard Instruments Corp., Groningen, The Netherlands).
Rotation vacuum concentrator Alpha RCV (Christ, Osterode, FRG).
LKB fraction collector Ultra Rac® 2211 (Pharmacia-LKB, Freiburg, FRG).
Shimadzu RF-551 fluorescence detector (Shimadzu, Duisburg, FRG).

c. 6.4 Analytical and Preparative HPLC
Apparatus
System A:
Analytical Chromatography
Analytical HPLC was performed on a HP 1090 M liquid chromatograph equipped with work station, diode array detector (DAD) and A/D-converter HP 35900C (Hewlett Packard, Waldbronn, FRG). The UV trace was recorded by the workstation. The radioactivity signal was monitored by the radioactivity detector Ramona® 5 and evaluated by the integration software of Raytest operated on a personal computer (Raytest, Straubenhardt, FRG). In addition, the radioactivity signal was digitalized by the A/D-converter and evaluated by the work station.
System B:

Preparative Chromatography
Preparative HPLC was performed by two Latek-p 402 pumps, wavelength detector and the radioactivity signal was monitored by the radioactivity detector Ramona® 5 and evaluated by the integration software of Raytest operated on a personal computer (Raytest, Straubenhardt, FRG).

c. 6.5 Recovery
Radioactivity of liquid samples was measured by liquid scintillation counting with automatic quench correction by the external standard channel ratio method at 13 °C using Ultima Gold® as scintillation cocktail.

c. 6.6 HPLC-14C
c. 6.6.1 Analytical HPLC-14C
The radioactivity signal was monitored by a Ramona 5, digitized by the A/D-converter and evaluated by the HP-Chemstation.
System A:
Column no. 503 : Nucleosil®100 C8 HD (3 µM), 75 x 2 mm
Column no. 301 : Nucleosil®100 C8 HD, 125 x 2 mm, particle size 5 µm with guard column Nucleosil®100 C8 HD (Macherey & Nagel, FRG).
Eluent : A = 50 mM ammonium acetate; : B = acetonitrile
Flow : 0.4 mL/min
Gradient : 0 min 10 % B
5 min 10 % B
30 min 35 % B
40 min 80 % B
42 min 80 % B
45 min 10% B
Equilibration time : 6 minutes
Injection volume : varying
Ramona cell : 50 µL

c. 6.6.2 Preparative HPLC-14C
The radioactivity signal was monitored by a Ramona 5.
System B:
Column, no. 300 : Nucleosil®100 C18 HD, 250 x 10 mm, particle size 5 µm (Macherey & Nagel, FRG).
Eluent : A = 50 mM formic acid ammonium salt; : B = acetonitrile
Flow : 4.0 mL/min
Gradient 9585-2 : 0 min 10 % B
5 min 10 % B
15 min 40 % B
20 min 40 % B
25 min 50 % B
30 min 80 % B
40 min 80 % B
42 min 10 % B

16
Gradient 9585-3 : 0 min 10 % B
3 min 10 % B
6 min 20 % B
30 min 45 % B
35 min 45 % B
40 min 80 % B
43 min 80 % B
45 min 10 % B
Equilibration time : 10 minutes
Injection volume : varying
Ramona cell : 200 µL

c. 6.6.3 Isolation and Purification of Metabolites from Human Liver Microsomes
All incubation procedures are already described in a previous report2 with the exception of the analytical HPLC, being columns no. 301 (125 x 2 mm) and 503 (75 x 2 mm) both with guard column. 10 incubation of 10ml each were performed in parallel.
The deproteinated incubations were combined and cleaned by solid phase extraction. The metabolites were then separated by preparative HPLC, and finally subjected to LC-14C-MS analysis, respectively, in order to assure identity and purity of the fractions. The entire procedure is shown in the flow chart below.

The procedure provided finally the following fractions:
fraction KNM... amount [µg] assignment 14C-[M+H]+
3100 I13 510 **M-1** 402
3100 I10 156 **M-2** 420
3100 I06 154 **M-3** 420
3100 M04 fr. 10 12.5 **M-4** 418
3100 M02 fr. 05 25.5 **M-5** 418

c. 6.7 Spectroscopic Methods
c. 6.7.1 HPLC-14C-MS/MS
The HPLC system consisted out of a Capillary LC-System 1100 (Agilent, Waldbronn, FRG), a column oven: Mistral Slave® (Spark, Emmen, Netherlands) with 6-ways-tandem-microvalve (SunChrom, Friedrichsdorf, FRG) and an A/D-converter HP 35900 C (Hewlett-Packard, Waldbronn, FRG). The employed Mass spectrometer was a Q-Star-Pulsar® mass spectrometer with API source operated in positive ion mode (Sciex, Toronto, Canada). The radioactivity signal was monitored by a Ramona 2000, digitized by the A/D-converter and evaluated by the HP-Chemstation and the MS computer.
System B:
Column : Nucleosil®100 C8 HD, 150 x 0.8 mm, particle size 3 µm (Grom Analytik + HPLC GmbH, FRG) with guard column Nucleosil® 100 C8 HD (Grom Analytik + HPLC GmbH, FRG).
Eluent : A = 10 mM ammonium acetate (+ raffinose pentahydrate, acarbose, cyclodextrine 10 mg/L of each, saccharose 4 mg/L)

: B = acetonitrile
Flow : 64 µL/min
Gradient : 0 min 0 % B
5 min 0 % B
30 min 15 % B
45 min 30 % B
47 min 90 % B
52 min 90 % B
54 min 0 % B
Equilibration time : 10 minutes
Injection volume : 25 µL
Ramona cell : 10 µL
Structure proposals are based on the data in the following file folders:
SMEK 1922I, SMEK 1922J
MS conditions were stored with the raw data.

c. 6.7.2 NMR Spectroscopy

1H and 2D-NMR spectra were recorded on an DRX 700 NMR spectrometer (Bruker, Rheinstetten, FRG) at 700 MHz and 25 °C using deutero-chloroform as solvent (99.96 % deuterium incorporation). The spectra were calibrated on C**H**Cl3 at □= 7.30 ppm.

c. 7. References

1 GERISCH M: Labeling Synthesis of artemisone with Carbon-14. Bayer Pharma Report PH-32886.
2 SCHMEER K: [14C]artemisone – Incubation with Liver Microsomes of Different Species Including Man – Elucidation of the Most Human like Animal. Bayer Pharma Report PH-32787.

2. Incubation Studies

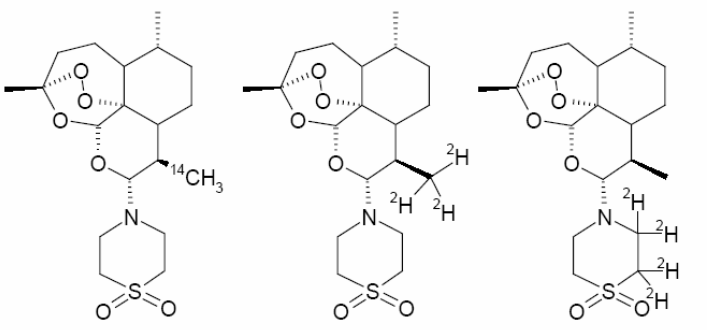
[^{14}C]BAY 44-9585 - Incubation with Liver Microsomes of Different Species Including Man - Elucidation of the Most Human like Animal

Abstract:
The metabolite profiles from incubations of [14C]-artemisone with liver microsomes from man (mix), Rhesus monkey, Beagle dog, Himalaya rabbit, Wistar rat, CD-1 mouse, and NMRI mouse were compared by LC-14C and LC-14C-UV-MS/MS analysis.

- Turnover after 30 min of incubation with NADPH2 +-regenerating system ranged between 21 % (female rat) and 97 % (Himalaya rabbit) of total radioactivity.
- Major phase I biotransformation reactions are: (1) Dehydrogenation in the thiomorpholine-dioxide moiety, leading to the metabolite **M-1**. (2) Mono-hydroxylation in the methyl-oxepane moiety, leading to the metabolite **M-2**. (3) Mono-hydroxylation in the methylcyclohexyl moiety, leading to the metabolite **M-3**.
- No hints for the presence of dihydroartemisinin (DHA) were found.
- Rhesus monkey, NMRI and CD-1 mouse reflected the phase I biotransformations in human liver microsomes at best and are therefore regarded as most human like animals. Himalaya rabbit covered all major and most minor phase I reactions as well, however in different quantities and can be also regarded as human like. Male Wistar rat is only partially human like, due to the low exposure of **M-1**. While the exposure of **M-3** was found to be low, metabolite **M-2** lacked completely in the pattern of dog microsomes. It can therefore not be regarded as human like.
- A clear quantitative and qualitative gender difference was observed between liver microsomal incubations from male and female Wistar rats.

a. Introduction

[14C]-Artemisone, [(3R,5aS,6R,8aS,9R,10R,12R,12aR)-3,6,9[14C]-trimethyl-decahydro-3,12-epoxy[1,2]dioxepino[4,3-i]isochromen-10-yl]thiomorpholine 1,1-dioxide (1), has been incubated with liver microsomes from Rhesus monkey, Beagle dog,

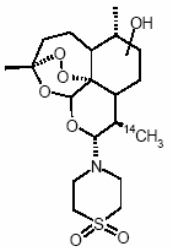
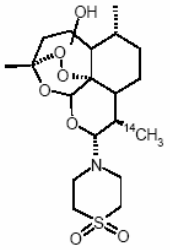
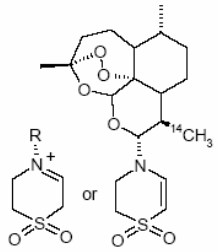
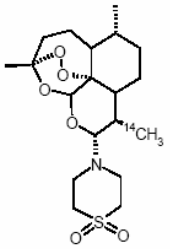
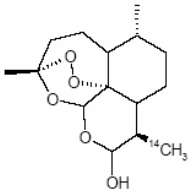


Himalaya rabbit, Wistar rat (male), NMRI mouse, CD-1 mouse, and man (mix) to elucidate the most human like animal with regard to phase I metabolism. The samples were analyzed by HPLC-14C-UV-MS/MS to compare the metabolite profiles. Mass spectrometric data refer to the smallest chemical entity of a given compound. Salt forms are not taken into consideration.

b. Results and Discussion

The proposed fragmentation pathway of the parent compound was established by comparing tandem-MS spectra of the non-labeled, the stably labeled2, 3 and the 14C-labeled compound. Enzymatic reactions were stopped at 10 and 30 minutes after start of incubation and the samples were analyzed by HPLC-14C-UV-MS/MS and, where necessary, with LC-14C-UVMS/MS/MS, respectively. **Table 1** and **Table 2** comprise the quantitative metabolite profiles, as established by HPLC-14C in **Figure 1**. A scheme of the assumed metabolic fate of the parent drug is shown below.

The 14C-peaks are given by their mass and retention time. The proposed structures for the metabolites are based on accurate mass determination and/or MS/MS spectra. Certain attention was paid to the possible formation dihydroartemisinin (DHA). The ion traces were reprocessed for the possible presence of this metabolite and compared with synthetic reference. However, no hints for the presence of dihydro-artemisinin (DHA) were found.

r_t (^{14}C) [min]	[^{14}C-M+H]$^+$	assignment	structure proposal
20.5	420	**M-3**	
24.3	420	**M-2**	
33.4	402	**M-1**	
36.5	404	**drug**	
		DHA (not observed)	

c. Conclusions

The metabolite profiles from incubations of [14C]BAY 44-9585 with liver microsomes from man (mix), Rhesus monkey, Beagle dog, Himalaya rabbit, Wistar rat, CD-1 mouse, and NMRI mouse were compared by LC-14C and LC-14C-UV-MS/MS analysis.

- Turnover after 30 min of incubation with NADPH2+-regenerating system ranged between 21 % (female rat) and 97 % (Himalaya rabbit) of total radioactivity.
- No hints for chemical degradation in buffer were found.

- No hints for NADPH2 +-regenerating system independent (non-CYP450/FMO) biotransformations, were found in liver microsomal incubations of all species.
- Major phase I biotransformation reactions are:
  i. Dehydrogenation in the thiomorpholine-dioxide moiety, leading to the metabolite **M-1**.
  ii. Mono-hydroxylation in the methyl-oxepane moiety, leading to the metabolite **M-2**.
  iii. Mono-hydroxylation in the methylcyclohexyl moiety, leading to the metabolite **M-3**.
- Minor phase I biotransformation reactions are:
  i. At least three other mono-hydroxylations. None of them seems to be hydroxylated in the 9-methyl-group.
  ii. One bis-hydroxylation.
- The metabolite pattern observed in incubations with human CYP3A4 corresponded well to that found in incubations with human liver microsomes4.
- No hints for the presence of dihydroartemisinin (DHA) were found, which is in good accordance to previous investigations5.
- Rhesus monkey, NMRI and CD-1 mouse reflected the phase I biotransformations in human liver microsomes best and are therefore regarded as most human like animals.
- Himalaya rabbit covered all major and most minor phase I reactions as well, however in different quantities and can be also regarded as human like.
- Male Wistar rat is only partially human like, due to the low exposure of **M-1**.
- While the exposure of **M-3** was found to be low, metabolite **M-2** lacked completely in the pattern of dog microsomes. It can therefore not be regarded as human like. Its role will be assessed by incubations with hepatocyte cultures.
- A clear quantitative and qualitative gender difference was observed between liver microsomal incubations from male and female Wistar rats. Liver microsomes from female Wistar rats formed only **M-3** in comparable amounts to males.

d. Tables

Table 1: [14C]-Artemisone

Metabolite profiles in incubations with liver microsomes of different species at two different time points after start of reaction (peaks that were only observed in the ion trace are not listed).

species			man (mix)			Rhesus monkey			Wistar rat (male)			Wistar rat (female)		
data file SMEK			1826S-02	1826S-03	1826S-04	1826S-05	1826S-06	1826S-07	1826S-08	1826S-09	1826S-10	1826S-26	1826S-27	1826S-28
time point			10 min	30 min	30 min stab.[1]	10 min	30 min	30 min stab.[1]	10 min	30 min	30 min stab.[1]	10 min	30 min	30 min stab.[1]
r_t (^{14}C) [min]	mass [^{14}C] [M+H]$^+$	interpre-tation	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]
13.3							2.8							
16.5	436			3.7		2.2	9.5			4.1				
19.1				5.0		3.6	11.3			6.2				
19.9	420	M-3	6.3	15.5		5.3	7.8		7.3	15.1		8.5	21.2	
20.5	420					13.2	12.2							
22.1	420													
24.3	420	M-2	9.8	18.6		38.4	44.8		33.1	50.2				
27.9	420									6.3				
33.4	402	M-1	29.2	34.2		11.1	9.1		6.6	5.2				
36.5	404	drug	54.6	23.1	100.0	26.3	2.5	100.0	53.0	13.1	100.0	91.5	78.8	100.0
metabolites balanced:			100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
metabolites assigned:			100.0	95.0	100.0	96.4	85.9	100.0	100.0	93.9	100.0	100.0	100.0	100.0

stab.[1]: stability control without regenerating system

Table 2: [14C]-Artemisone

Metabolite profiles in incubations with liver microsomes of different species at two different time points after start of reaction (peaks that were only observed in the ion trace are not listed).

species			NMRI mouse			Beagle dog			Himalaya rabbit			CD-1 mouse (male)			drug
data file SMEK			1826S-14	1826S-15	1826S-16	1826S-17	1826S-18	1826S-19	1826S-20	1826S-21	1826S-22	1826S-11	1826S-12	1826S-13	1826S-23
time point			10 min	30 min	30 min stab.[1]	10 min	30 min	30 min stab.[1]	10 min	30 min	30 min stab.[1]	10 min	30 min	30 min stab.[1]	30 min stab.[2]
r_t (^{14}C) [min]	mass [^{14}C] [M+H]$^+$	interpre-tation	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]
13.5										3.1					
16.5	436								3.7	10.1					
19.1										5.5		7.8	2.9		
19.9	420	M-3		9.0		5.2	6.6		19.3	24.2			8.0		
20.5	420		9.6	18.6		15.9	27.7		15.7	18.4			11.1		
22.1	420								5.9	4.0					
24.3	420	M-2	13.6	26.4					27.1	27.8		16.7	34.2		
27.9	420			1.9									5.0		
33.4	402	M-1	7.3	13.2		17.0	34.1		9.1	3.6		11.5	15.1		
36.5	404	drug	69.5	30.9	100.0	61.9	31.6	100.0	19.2	2.7	100.0	64.0	23.7	100.0	100.0
metabolites balanced:			100.0	100.0	100.0	100.0	100.0	100.0	100.0	96.2	100.0	100.0	100.0	100.0	100.0
metabolites assigned:			100.0	100.0	100.0	100.0	100.0	100.0	100.0	90.7	100.0	92.2	97.1	100.0	100.0

stab.[1]: stability control without regenerating system

stab.[2]: stability control buffer

Table 3: [14C]-Artemisone

Incubation with liver microsomes of different species (experimental data), HPLC analysis and recovery.

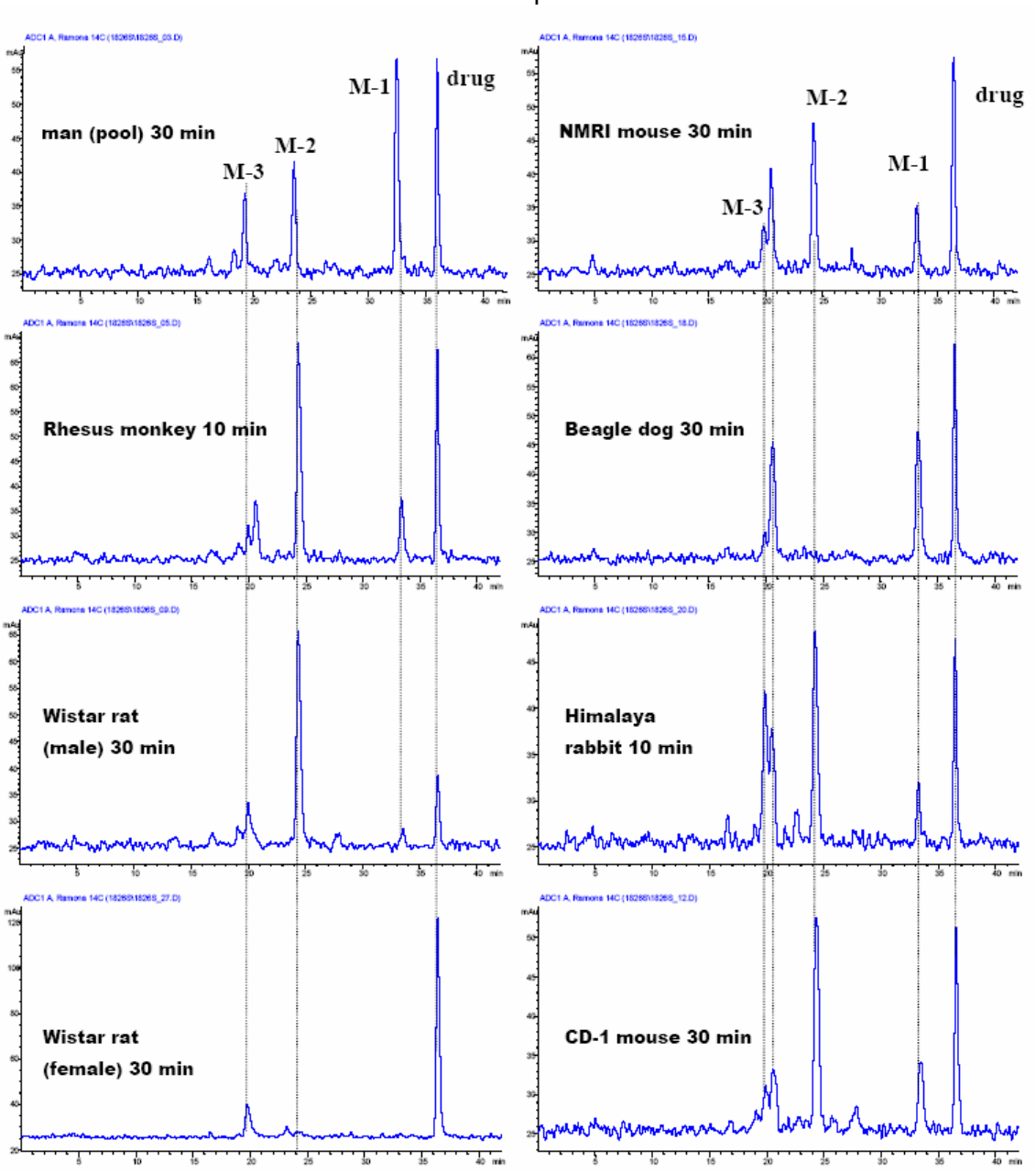
Time points [min]	Species	Sex	Batch	Incubation file SMEK	LC-MS and LC data file SMEK	Recovery [%]
10 min	man, 452161 (mix) *			1826R01	1826S02	110
30 min				1826R02	1826S03	102
30 min (stab. control)				1826R03	1826S04	109
10 min	monkey	f	Pt.1/97	1826R04	1826S05	105
30 min	(Rhesus)			1826R05	1826S06	99
30 min (stab. control)				1826R06	1826S07	102
10 min	rat	m	RLM-Wistar-m-P1/2002	1826R07	1826S08	108
30 min	(Wistar)			1826R08	1826S09	95
30 min (stab. control)				1826R09	1826S10	108
10 min	mouse	m	MLM-CD1-m-P1/2002	1826R10	1826S11	100
30 min	(CD-1)			1826R11	1826S12	103
30 min (stab. control)				1826R12	1826S13	104
10 min	mouse	m	MLM-NMRI-m-P1/2002	1826R13	1826S14	90
30 min	(NMRI)			1826R14	1826S15	106
30 min (stab. control)				1826R15	1826S16	109
10 min	dog	f	DL1	1826R16	1826S17	111
30 min	(Beagle)			1826R17	1826S18	107
30 min (stab. control)				1826R18	1826S19	82
10 min	rabbit	f	RBLM-Himalaya-f-P1/2002	1826R19	1826S20	85
30 min	(Himalaya)			1826R20	1826S21	83
30 min (stab. control)				1826R21	1826S22	81
10 min	rat	f	RLM-Wistar-w-P1/2002	1826R25	1826S26	109
30 min	(Wistar)			1826R26	1826S27	107
30 min (stab. control)				1826R27	1826S28	112

* human liver microsomes 452165 lot:22 were purchased from Gentest, Woburn, USA

e. Figures

Figure 1: [14C]-Artemisone

Radio-chromatograms from incubations of [14C]BAY 44-9585 with liver microsomes of different species.



f. Material and Methods

f.1 Labeled Drug

The 14C-labeled drug, batch no. : GCM1330-1-01A specific activity : 2.42 MBq/mg, was synthesized by Dr. M. Gerisch, BHC-PH-PD P MIC, Bayer AG, Wuppertal, FRG1; the 2H3-labeled drug, batch no. : L02/02, was synthesized by Prof. Haynes, Hongkong Univ. of Science and Technology (2); the 2H4-labeled drug, batch no. : u-a was synthesized by Dr. Wolfgang Calaminus, ZF-SF-TEC (Bayer Uerdingen) (3).

f.2 Reagents and Equipment

Chemicals

di-Potassium hydrogen phosphate no. 5101; Magnesiumchloride hexahydrate for analysis no. 5833; Saccharose no. 7653; Raffinose pentahydrate no. 1.07419 (E. Merck, Darmstadt, FRG); Acarbose (GT-Ware 1083/95HF1-3, Lab. Dr. Lenz, Bayer AG, Wuppertal, FRG); □-Cyclodextrine no. 28705 (Fluka, Buchs, Switzerland); Formic acid 98 - 100 % R.G. no. 33015; Ammonium acetate R.G. no. 32301; Water Chromasolv® no. 34877 Acetonitrile Chromasolv® no. 34851 (Riedel de Haen, Seelze, FRG); EDTA no. 11278; NADP no. 49207744; Glucose 6-Phosphate Dehydrogenase no. 85913432 (Roche Diagnostics GmbH, Mannhein, FRG); Glucose 6-Phosphate no. 92338429 (Roche Diagnostics GmbH, Mannhein, FRG); Ultima Gold® high flash point scintillation cocktail (Packard Instrument BV Chemical Operations, Groningen, The Netherlands).

Apparatus

System A:

Capillary LC-System 1100 (Agilent, Waldbronn, FRG).
Column oven: Mistral Slave® (Spark, Emmen, Netherlands).
A/D-converter HP 35900 C (Hewlett-Packard, Waldbronn, FRG).
Radioactivity monitors Ramona® 2000 connected to HP-Chemstation (Raytest, GmbH, Straubenhardt, FRG).
API 3000 triple stage mass spectrometer with API source (Sciex, Toronto, Canada).

System B:

Column oven: Mistral Slave® (Spark, Emmen, Netherlands), with 6-ways-tandem-microvalve (SunChrom, Friedrichsdorf, FRG).
A/D-converter HP 35900 C (Hewlett-Packard, Waldbronn, FRG).
Radioactivity monitor Ramona® 2000 connected to HP-Chemstation (Raytest, GmbH, Straubenhardt, FRG).
Capillary LC-System 1100 (Agilent, Waldbronn, FRG).
Q-Star-Pulsar® mass spectrometer with API source (Sciex, Toronto, Canada).
5417C centrifuge (Eppendorf AG, Hamburg, FRG).
Thermomixer comfort (Eppendorf AG, Hamburg, FRG).
Vacuum concentrator Alpha RVC with ice condenser L-1 (Christ, Osterode, FRG).

f.3 Recovery

Radioactivity of liquid samples was measured by liquid scintillation counting with automatic quench correction by the external standard channel ratio method at 13 °C using Ultima Gold® as scintillation cocktail.

f.4 HPLC-14C-MS/MS

The radioactivity signal was monitored by a Ramona 2000, digitized by the A/D converter and evaluated by the HP-Chemstation and the MS computer.

System B:

Column : Nucleosil®100 C8 HD, 75 x 0.8 mm, particle size 3 µm (Grom Analytik + HPLC GmbH, FRG) with guard column Nucleosil®100 C8 HD (Grom Analytik + HPLC GmbH, FRG).
Eluent : A = 10 mM ammonium acetate (+ raffinose pentahydrate, acarbose, □-cyclodextrine 10 mg/L of each, saccharose 4 mg/L)
: B = acetonitrile
Flow : 64 µL/min
Gradient : 0 min 10 % B
5 min 10 % B
30 min 35 % B
40 min 80 % B
42 min 80 % B
Equilibration time : 8 minutes
Injection volume : 20 µL
Ramona cell : 10 µL
Structure proposals are based on the data in the following file folders:
SMEK 1826N, SMEK 1826Q, SMEK 1826S, SMEK 1827C, SMEK 1827D, SMEK 1827E, SMEK 1827L.
MS conditions were stored with the raw data (**Table 3**).

f.5 Animals

Dog : female Beagle (body weight: approx. 9.2 kg), Bayer AG, FRG
Monkey : female Rhesus (body weight: about 3.5 – 4 kg), Supplier: OSIC, The Oriental Scientific Import + Export Cooperations, Republic of China
Mouse : male CD-1, Breeder: Charles River, Sulzfeld, FRG; male NMRI
Breeder: Harlan/Winkelmann, Borchen, FRG
Rabbit : female Himalaya: CH (body weight: approx. 2 – 3 kg), Bayer AG, FRG
Rat : female and male Wistar (body weight: 180 – 200 g), Breeder: Harlan/Winkelmann, Borchen, FRG

f.6 Preparation of Liver Microsomes from Animal Species

Human liver microsomes 452161 lot:22 were purchased from Gentest, Woburn, USA.
Liver microsomes from rhesus monkey were prepared as follows. Livers were removed and weighed. All further steps were performed at 4 °C. A 30 % homogenate in 50 mM HEPES buffer pH 7.4 was prepared. By centrifugation at 9.000 x g (20 min) cellular debris and mitochondria were removed. The remaining supernatant was centrifuged at 105.000 x g (60 min). After removing the cytosol the microsomal pellet was suspended in 50 mM HEPES buffer pH 7.4 and stored at –75 °C after freezing in liquid nitrogen.
All other liver microsomes were prepared according to SOP6.

species	batch	CYP P 450 [nmol/mL]	protein [mg/mL]	added microsomes [µL/mL incubation]
rat, male	RLM-Wistar-m-P1/2002	n.d.	20	25
rat; female	RLM-Wistar-w-P1/2002	n.d.	20	25
dog, female	DL1	10.8	20	25
mouse (CD-1), male	MLM-CD1-m-P1/2002	n.d.	20	25
mouse (NMRI), male	MLM-NMRI-m-P1/2002	n.d.	20	25
rabbit, female	RBLM-Himalaya-w-1/2002	n.d.	20	25
monkey, female	Pt. 1/97	15.7	20	25
human pool	452161 lot:22	7.2	20	25

f.7 Incubation with Liver Microsomes of Different Species

The experimental data are summarized as follows:
Files : SMEK1826P, SMEK 1826R, SMEK 1826T
NADPH2+-regenerating system : Buffer : 50 mM K2HPO4 + 1 mM EDTA (adjusted on pH 7.4) at 37 °C
NADP : 1 mM
Glucose-6-Phosphate : 5 mM
Glucose-6-Phosphate Dehydrogenase: 1.5 U/mL
Substrate : [14C]BAY 44-9585 (batch no. GCM 1330-1-01A),
spec. activity: 2.42 MBq/mg) or [2H4]BAY 44-9585
or [2H3]BAY 44-9585 in acetonitrile/water (1:1),
20 µM final concentration
8 µL were added to the solution
Protein concentration : 0.5 mg/mL
Incubation volume : 1 mL
Incubation conditions : shaking at 37 °C and 1200 RPM
Time points : 10 min and 30 min
Terminated : addition of 1 mL acetonitrile to 1 mL incubation
Preparation for LC-MS : 250 µL of the well centrifuged supernatant was concentrated to 70 µL

g. References

1 GERISCH M: Labeling Synthesis of BAY 44-9585 with Carbon-14. Bayer Pharma.
2 Richard K.Haynes, Sum Wai Lam, Ho Ning Wong, Hing Wo Tsang: "Procedure for the Optimized Preparation of 15-triDeutero-Artemisone (BAY 44 9585)". Hong Kong University of Science and Technology 2003.
3 CALAMINUS W: "Synthese von 2`2`3`3`-Tetradeutero-Artemisone (BAY 44-9585) als internem Standard für quantitative MS- und NMR-Untersuchungen". Bayer AG, ZF-SY-Tec (2002).
4 RADTKE M: Identification of CYP-Isoforms involved in the biotransformation of [14C]BAY 44-9585. Bayer Pharma.
5 A & M, BERGHEIM: BAY 44-9585: Determination of the Metabolite DHA in Human Liver Microsomes by Means of LC-MS/MS. Study A&M 01-013, Short Report (2001).
6 GÄFKE D: Herstellung von Lebermikrosomen SOP.