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Supplementary Material

Discovery, Synthesis, Activities, Structure-Activity Relationships, and Clinical Development of Combretastatins and Analogs as Anticancer Drugs. A Comprehensive Review*

Sheo B. Singh, PhD, FRSC

Charles and Dana Research Institute for Scientists Emeriti (RISE), Drew University, Madison, NJ 07940, Department of Chemistry and Chemical Biology, Stevens Institute of Technology, Hoboken NJ 07030, and SBS Pharma Consulting LLC, Edison NJ 08820

Correspondence: sbsingh101@gmail.com; sheo.singh@stevens.edu

* Dedicated to my Late mentors- Prof. G. R. Pettit (Arizona State University, Tempe, AZ), Prof. K. H. Overton (University of Glasgow, Glasgow, UK), Dr. R. S. Thakur (CSIR CIMAP, Lucknow, India)

ISOLATION OF COMBRETASTATINS (1-22)

Combretastatin (**7**) was first isolated by bioactivity guided fractionation of a methanol-CH_2Cl_2 extract of 55 kg dried leaves, fruits, and chipped twigs of *C. caffrum* collected from South Africa in 1979 under a collaborative agreement between the United States National Cancer Institute (NCI) and Department of Agriculture (USDA) for discovery of anticancer agents. While this extract was highly active against the PS cell line as well as the P388 (PS) leukemia *in vivo* assays, a new astrocyte reversal assay (9ASK) assay was used for bioassay guided fractionation for this purification. After solvent partitioning using hexane and CCl_4, the active fraction was extracted by CH_2Cl_2. A 60% aliquot was fractionated sequentially on Sephadex LH20 and silica gel columns followed by medium pressure chromatography on a silica gel Lobar column affording crystalline (-)-combretastatin **7** (0.452 g, $1.4x10^{-3}$% dry plant weight yield).[1,2] This compound accounted for 9ASK activity (71-90% astrocyte reversal at 100-1 mg/mL and 51-70% as 0.1 mg/mL doses) and showed potent PS cell line activity (ED_{50} 0.03 μM) but surprisingly it was devoid of the *in vivo* efficacy observed originally in the starting CH_2Cl_2 soluble fraction, thus requiring fresh isolation efforts using the PS assay.[3,4]

In the last week of 1983, when the author of this review joined the laboratories of Prof. Pettit at Arizona State University, he was assigned the isolation of the PS active component(s) from *C. caffrum*. The author's work started with 77 kg of chipped bark which was collected at the same time in 1979 as the leaves and twigs which were used for the first isolation described above. The plant material was extracted with methanol- CH_2Cl_2 (1:1) twice for 10 days each at ambient temperature providing an extract (1.41 kg) which exhibited the original *in vitro* and *in vivo* PS activity (Figure 2). This extract was partitioned with hexane and CH_2Cl_2 as described in the Figure 2. Size exclusion chromatography of the active CH_2Cl_2 extract (683.4 g, ~98g each x 7 columns) led to the two active fractions A (28.6 g, later eluting fraction, elution with 4-5 column volume) and B (30.6 g, earlier eluting fraction, elution with 3-4 column volume).[3-11] Included here is a consolidated summary of the chronological steps of bioassay guided fractionation for the isolation of the compounds including an isolation scheme (Figure 2), which was compiled from eight publications[3-10] reporting specific classes of compounds, as well as authors personal unpublished notes.

Partition chromatography on Sephadex LH 20 using hexane-toluene-methanol (3:1:1) of the fraction A provided four main active fractions weighing 1.97, 0.54, 0.96 and 4.9 g. Crystallization of the 4.9 g fraction gave a weakly active crystalline dihydrophenanthrene **15** (500 mg, 6.7×10^{-4}%, ED_{50} 7.28 μM) (Table 1),[6] the first compound isolated from the new isolation efforts (Figure 2). Silica gel chromatography of the mother liquor afforded phenanthrene **19** (66.9 mg, 1.1×10^{-4}%, PS ED_{50} 6.37 μM),[6] another weakly active compound. Silica gel chromatography of the 0.54 and 0.96 g fractions yielded combretastatin D-1 (**21**) (180 mg, 2.6×10^{-4}%, PS ED_{50} 10.57 μM)[5] and combretastatin B-4 (**11**) (35.8 mg, 4.6×10^{-5}%, PS ED_{50} 6.20 μM).[7] Finally, the last 1.97 g fraction was dissolved in hexane-toluene-methanol (3:1:1) and filtered affording pure combretastatin C-1 (**20**) (11 mg, 1.4 x 10-5%, PS ED_{50} 7.00 μM)[9] as an orange solid, and Sephadex LH20 chromatography of the filtrate on a long column using the same 3:1:1 solvent followed by silica gel chromatography led to isolation of pure combretastatin D-2 (**22**) (5.8 mg, 7.5×10^{-6}%, PS ED_{50} 17.56 μM)[10] and potent combretastatin A-2 (**2**) (442 mg, 5.8×10^{-4}%, PS ED_{50} 0.09 μM) (Figure 2).[4]

Similar partition chromatography of the fraction B on Sephadex LH20 with 3:1:1 solvent also led to four main active fractions weighing 1.46, 2.41, 8.11 and 1.57 g. Multiple sequential silica gel chromatographic efforts of the 1.57 g fraction afforded highly potent combretastatin A-3 (**3**) (480 mg, 6.3 x 10-4%, PS ED_{50} 0.09 μM)[4] as a major component of the fraction along with moderately potent combretastatin B-3 (**10**) (97 mg, 1.3 x 10^{-4}%, PS ED_{50} 1.32 μM),[7] and weakly active dihydrophenanthrene **17** (86.4 mg, 1.1 x 10^{-4}%, PS ED_{50} 8.94 μM).[6] Likewise, multiple sequential silica gel chromatography of the largest 8.11 g fraction afforded the highest yield of the active components, combretastatin A-1 (**1**) (700 mg), along with another 138 mg from side fractions, giving a total of 838 mg, (1.1 x 10^{-3}%, PS initial ED_{50} 2.98 μM,[3]; more recently ED_{50} 0.6 μM from fresh solution),[3,12] along with combretastatin B-1 (**8**) (39.6 mg, 5.1 x 10^{-5}%, PS ED_{50} 5.09 μM)[3] and combretastatin B-2 (**9**) (51.7 mg, total 370.7 mg, 4.8 x 10^{-4}%, PS ED_{50} 1.05 μM).[4] A single silica gel chromatography of the 1.46 g fraction led to weakly active compounds, a dihydrostilbene **12** (102 mg, 1.5 x 10^{-4}%, PS ED_{50} 5.90 μM),[7] and a dihydrophenanthrene **16** (50 mg, 10^{-5}%, PS ED_{50} 8.86 μM).[6] Lastly, silica gel chromatography of the 2.41 g fraction led to the isolation of the most abundant weakly active compound, dihydrostilbene **13** (1.15 g, 1.5 x 10-3%, PS ED_{50} 9.68 μM)[7] along with dihydrophenanthrene **18** (10 mg, 1.3 x 10^{-5}%)[6], while the 0.56 g fraction contained many compounds, including the most potent compound, combretastatin A-4 (**4**). This fraction was further chromatographed on another silica gel column leading to the purification of a potent dihydrostilbene **14** (10 mg, 1.3 x 10^{-5}%, PS ED_{50} 0.87 μM)[7] and yet another 26.4 mg fraction containing an inseparable mixture of combretastatin A-4 (**4**), A-5 (**5**) and A-6 (**6**). Separation of these compounds using contemporary (1984-1986) separation media including finer silica gel employing PLC and HPLC failed. Hydrogenation of small amount of the mixture led to a simpler inseparable mixture. 400 MHz ^{1}H NMR analysis of the mixture indicated a positional isomeric stilbene mixture including *cis* and *trans* isomers which resulted in a regioisomeric dihydrostilbene mixture. At this point, the highly potent (0.034 μg/mL) and chromatographically homogeneous fraction (7.2 mg) was set aside in pursuit of alternative purification methods (Figure 2).

In late 1986, after working on combretastatins (**1**-**3**, **8**-**22**) for more than a year, including total synthesis, biological evaluations, and SAR, and at the culmination of the program, the work on this 7.2 mg abandoned fraction was reinitiated, facilitated by two facts. Firstly, the activity of this fraction was as potent as the most active compound discovered till date, i.e., combretastatins A-1, indicating that one of the three compounds in the mixture may be more potent than any of the other compounds, unless all three were equally potent. In either event, the resolution of the compounds of the fraction was of high interest. Secondly and fortuitously, synthetic work provided clear evidence of the easy separability of TBS ether derivatives of *cis* and *trans*-stilbenes. Therefore, in a last-ditch effort, the TBS ethers of 7.2 mg fraction was prepared. Gratifyingly, the TBS ethers were easily separated by silica gel PLC affording pure TBS ethers of combretastatin A-4 (**4a**) (3 mg, equivalent to 2.2 mg of **4**, 2.9x10^{-6}%), combretastatin A-5 (**5a**) (2 mg, equivalent to 1.5 mg of **5**, 1.9x10^{-6}%), and *trans* combretastatin A-6 (**6a**) (1.5 mg, equivalent to 1.1 mg of **6**, 1.4 x10^{-6}%) (Figure 2).[8,11] Purified **5a** upon storage even in the solid state produced **6a** suggesting that the *trans*-stilbene **6a** may be an artifact.[11] Desilylation of **4a**, **5a**, and **6a** gave **4** (PS initial ED_{50} 0.003 μM,[8,11] more recent ED_{50} 0.00095 μM),[8,11,12] **5** (PS ED_{50} 2.84 μM)[11] and **6** (PS ED_{50} 56.96 μM)[11], respectively. It is evident that the discovery of the

minute amount of the most interesting compound, combretastatin A-4 (**4**), would have not been possible if the isolation work-up had not begun with a large amount of plant material (Figure 2). The two isolation efforts from the extracts of the two distinct plant parts did not lead to the isolation of any overlapping compounds, despite combretastatin (**7**) being active in both 9ASK and PS *in vitro* assays. Whether it was due to the strict compartmented distribution of these compounds in the specific plant parts or something else remains unresolved, as no such comparative analytical work was performed at the time and is not envisaged now.

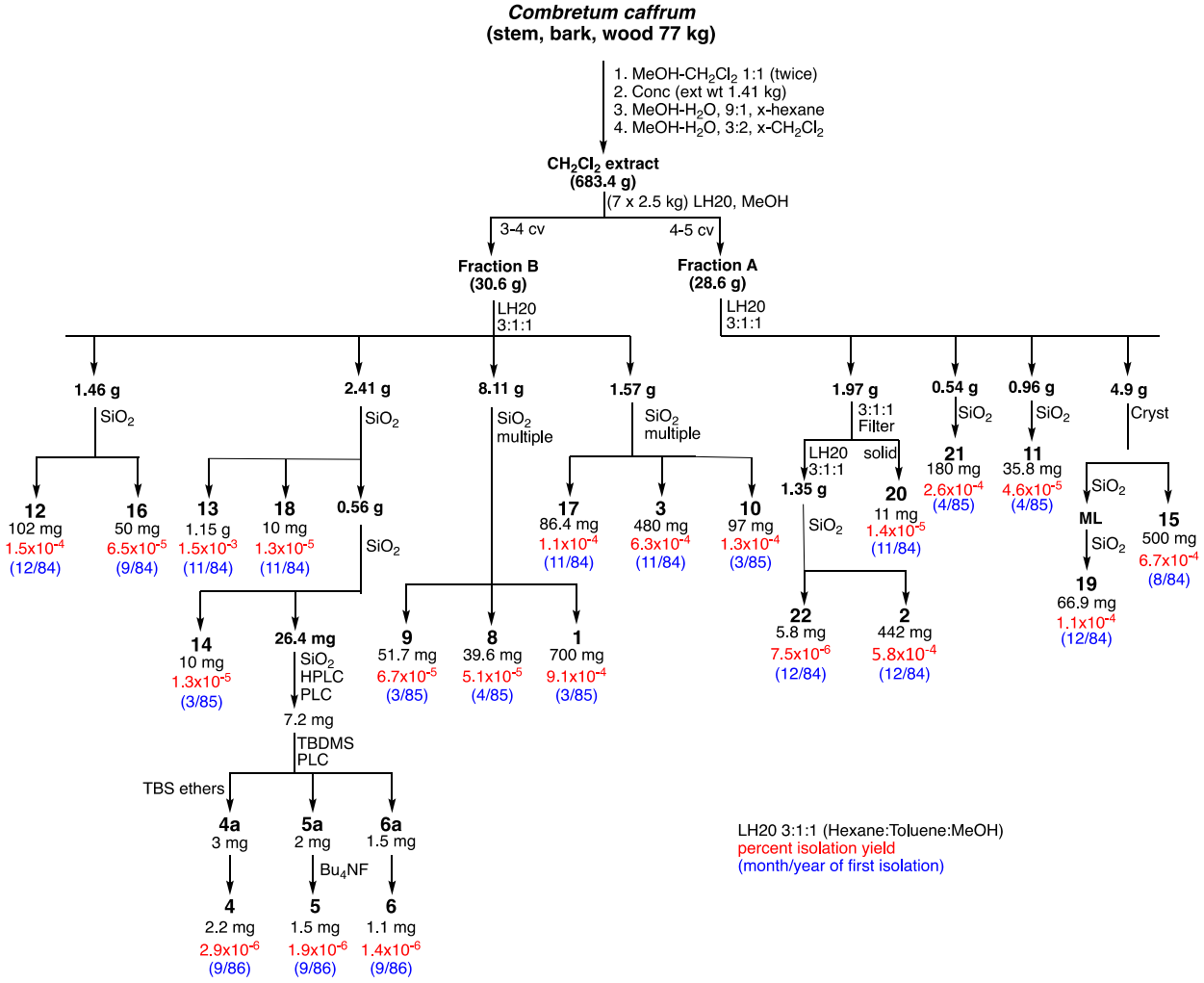

Figure 2. Composite scheme of the isolation of combretastatins from the stem bark extract of *C. caffrum* including the amounts of the isolated materials from the main active fractions and the percent isolation yields based on dried plant material, and the isolation month and year. Additional amounts of **1** (138 mg), **3** (74 mg), **9** (319 mg), **14** (30 mg), **17** (196.5 mg) were isolated from side fractions not included above.

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