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Association (micellization) and partitioning of aglycon triterpenoids

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ABSTRACT

Micellization and solution properties of the aglycon triterpenoids asiatic acid (AA) and madecassic acid (MA) were examined experimentally and in computational simulations. AA and MA belong to the large class of bioactive aglycon triterpenoids, for which limited physicochemical data are available. In this study, solubility, partition coefficient, critical micelle concentrations (CMC), and surface tensions of AA and MA were measured. Reverse phase HPLC data, supported by dye probe experiments and drop shape analysis, showed the CMC in phosphate buffered saline (PBS) to be 15 ± 2 μM, and 86 ± 9 μM for AA and MA, respectively. The surface tensions of AA and MA in PBS were 64.1 and 64.4 mN/m, respectively. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry indicated the aggregation numbers of AA and MA to be 5 to 7. Molecular dynamics simulations confirmed that molecular association could occur between 5 and 7 molecules in solution. The IC_{50} of AA and MA on human small cell carcinoma and human glioblastoma cell lines was 25 ± 5 μM and 66 ± 13 μM, respectively. The IC_{50} is within the range of calculated CMC of AA and MA in bioassay media, suggesting that the micellar aggregates may lead to their cytotoxicity.

1. Introduction

Over 4000 aglycon triterpenoids have been isolated from various plants and many have been suggested to have therapeutic applications, including anti-inflammatory and wound-healing properties [1,2]. Surprisingly, many biochemical mechanisms of bioactivity for these compounds have been proposed in the literature, without full consideration of their fundamental properties (e.g. solubility, partition coefficient, and critical micelle concentration, etc.). This is particularly notable because many aglycon triterpenoids contain hydrophobic and hydrophilic regions, which therefore might associate to form micelles or soluble aggregates.

While many of these compounds have been identified, obtaining large quantities of them in sufficient purity for experimentation is often difficult and time consuming. Furthermore, these compounds are mainly soluble in organic solvents or in organic/aqueous media, complicating the application of many ordinary techniques for physicochemical analysis that require mainly aqueous media. To address these problems, a reverse phase HPLC (RP-HPLC) procedure was developed to determine CMC. The procedure requires only a few milligrams of aglycon triterpenoid sample in organic/aqueous media.

Asiatic acid (AA) and madecassic acid (MA) are the most common aglycon triterpenoids of the commercially and traditionally popular medicinal herb *Centella asiatica*. They are frequently used for various therapeutic effects. Extracts such as TECA (titrated extract of *Centella asiatica*) [1] and dried aerial parts of these plants are available over the counter as therapeutics, frequently with the claim that their bioactive principles are triterpenoids such as AA or MA. Recent studies have also suggested that AA may have wide ranging multi-functional effects [3] and may even be an apoptotic anti-cancer agent [4–7], further bolstering claims of *Centella asiatica*'s medicinal properties.

AA and MA have chemical structures that are similar to those of bile acids well known surface active agents in physiological systems [8]. Bile acids, have a tetracyclic "steroid" skeleton, while AA and MA are pentacyclic [9]. These compounds are also semi-rigid, containing both hydrophobic and hydrophilic segments grouped mostly on opposite sides of the molecule (Fig. 1) [10,11]. The structural parallels between bile acids and aglycon triterpenoids suggest that the latter may also be surface-active and self-assemble into micelles.

The critical micelle concentration (CMC), partition coefficient, and solubility of AA and MA were determined. Molecular dynamics simulations complimented these experiments by modeling the

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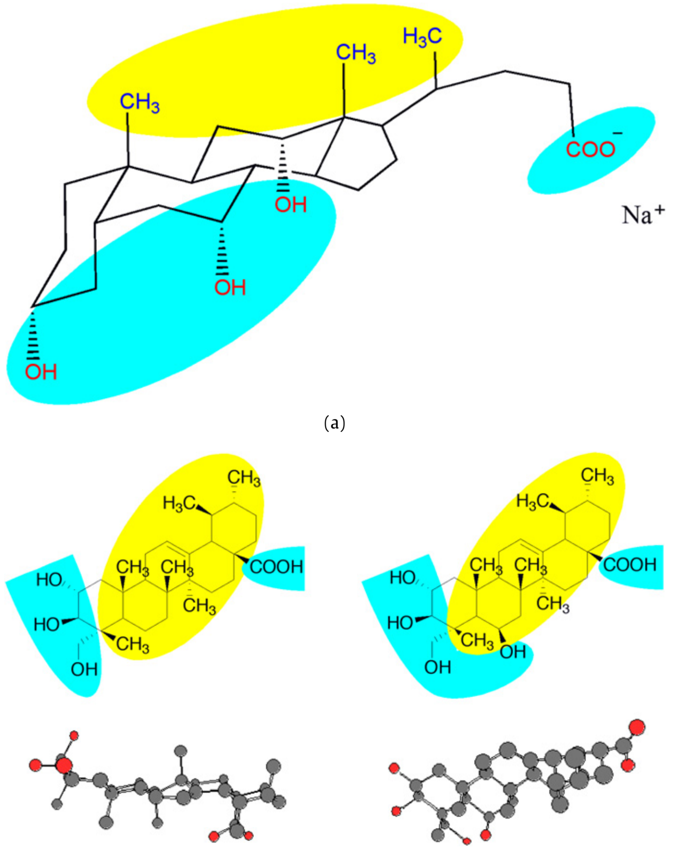

Fig. 1. (a) Structure of a bile salt [37]; (b) Structures of asiatic (left) and madecassic acid (right). The three-dimensional structures were obtained by energy minimization using Chem 3D Ultra (CambridgeSoft, Cambridge, MA, USA).

aggregation and interactions of these molecules in solution. IC_{50} were also determined on human small cell carcinoma (DMS114) and human glioblastoma cells (HTB-14) to compare to CMC. This is the first study to report the solution and partition behaviors of AA and MA.

2. Experimental

2.1. Reagents and solutions

AA and MA samples were provided by MMP Inc. (International Development and Manufacturing, South Plainfield, NJ, USA). The purity and identity of the samples were confirmed by analytical HPLC and MS. Samples were further purified to better than 99% purity by semi preparatory HPLC. Phosphate Buffered Saline solution (1 × PBS, pH 7.2, Mediatech, Herndon, VA) was used in all experiments. All other chemicals were reagent grade (Mallinckrodt, St. Louis, MO).

2.2. Solubility

Calibration curves for the solubility were constructed from RP-HPLC data. AA and MA samples were prepared by serial dilution of a stock solution of known concentration in methanol. The saturated solutions of AA and MA were made in phosphate buffered saline (PBS) and shaken overnight. Each saturated solution was centrifuged, and 1 mL of the supernatant was diluted to 10 mL with methanol. The concentration of the diluted solution (and hence the solubility) was determined by RP-HPLC using a calibration curve constructed by analysis of known concentrations of standard triterpenoid solutions.

*2.3. Partition coefficient (*log *K)*

Partition coefficients were determined [12] by dissolving 1 mg of AA or MA in 1 mL of octanol and 1 mL water. The solution was then shaken overnight. Both the upper and lower liquid phases were analyzed by RP-HPLC to determine the quantity of AA and MA in each phase. The partition coefficient was calculated by computing the ratio of the concentration in octanol to the that in water [13,14] as determined by HPLC peak areas. The hydrophobic–lipophilic balance (HLB) of each triterpenoid was estimated using the software Molecular Modeling Pro (Norgwyn Montgomery Software, North Wales, PA).

2.4. Reverse phase analytical HPLC assay

Solutions of various concentrations below and above the CMC of AA and MA were made in methanol, PBS, or 1% DMSO/ethanol in PBS. Each 20 μL sample was passed through an HPLC system (D-7000 HPLC System, Hitachi Instruments, Inc., San Jose, CA) at 210 nm with a 5 μm, 100 A, 150 mm L × 3.19 mm ID Delta-PAK C4 column (Waters, Milford, MA) at a flow rate of 1 mL/min. The mobile phase was 0.05% H_3PO_4 in water (A) and acetonitrile (B). A gradient table was developed for separation of compounds. The peak area versus triterpenoid concentration was plotted. Each solution was prepared using serial dilutions of stock solutions in designated solvents.

2.5. Preparatory HPLC procedure for purification

Solutions containing compounds were further purified with an HPLC system (Polaris System, Varian Inc., Palo Alto, CA) with a Platinum EPS C18 column, 10 μm, 250 mm L × 10 mm ID (Altec, Deerfield, MI). The compounds were eluted with a gradient system: 0.1% TFA in water (solvent A) and acetonitrile (solvent B), at a flow rate of 5 mL/min and detected at 210 nm. Heart cut fractions were collected and lyophilized to obtain the purified compound as a dry, white, powder of greater than 99% purity.

2.6. Drop shape analysis

Surface tensions and CMC were determined by the drop volume method [15–17]. Saturated solutions of AA and MA were made in PBS. AA or MA solutions were prepared by diluting the supernatant from a saturated stock solution. The diluted concentrations of AA or MA were selected to ensure that measurements were made at concentrations both below and above the CMC. Samples were loaded onto a syringe and analyzed by a G10/DSA10 Drop Shape Analysis System (Kruss GmbH, Hamburg, Germany) to determine the surface tensions. Measurements were taken after the drops equilibrated for 5 min.

2.7. Dye probe method with Nile red

The absorption of AA and MA with Nile red in PBS was measured at 220 nm with a UV spectrophotometer (Beckman DU 530 Life Science, Fullerton, CA). Each sample containing 3 mM Nile red was shaken overnight. The optical densities (OD) at 590 nm were measured for several dilutions of AA and MA.

2.8. Aggregation number

The aggregation numbers for AA and MA were estimated using matrix assisted laser desorption ionization (MALDI) spec-