

Interfacial properties of most monofluorinated bile acids deviate markedly from the natural congeners: studies with the Langmuir-Pockels surface balance

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Abstract We characterized the air-water interfacial properties of four monofluorinated bile acids alone and in binary mixtures with a common lecithin, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), using an automated Langmuir-Pockels surface balance. We compared 7 α -fluoromurocholic acid (FMCA), 7 α -fluorohyodeoxycholic acid (FHDCa), 6 α -fluoroursodeoxycholic acid (FUDCA), and 6 α -fluorochenodeoxycholic acid (FCDCa) with their natural dihydroxy homologs, murocholic acid (MCA), hyodeoxycholic acid (HDCA), ursodeoxycholic acid (UDCA), and chenodeoxycholic acid (CDCA). For further comparison, two trihydroxy bile acids, 3 α ,6 β ,7 α -trihydroxycholanoic acid [α -muri-cholic acid (α -MCA)] and 3 α ,6 α ,7 β -trihydroxycholanoic acid [ω -muri-cholic acid (ω -MCA)], with isologous OH polar functions to FMCA and FUDCA were also studied. Pressure-area isotherms of MCA, HDCA, UDCA, CDCA, and FMCA displayed sharp collapse points. In contrast, FHDCa, FUDCA, and FCDCa formed monolayers that were less stable than the trihydroxy bile acids, displaying second-order phase transitions in their isotherms. All natural and fluorinated bile acids condensed mixed monolayers with POPC, with maximal effects at molar bile acid concentrations between 30 and 50 mol%. Examination of molecular models revealed that the 7 α -F atom of the interfacially stable FMCA projects away from the 6 β -OH function, resulting in minimal steric interactions, whereas in FHDCa, FUDCA, and FCDCa, close vicinal interactions between OH and F polar functions result in progressive bulk solubility upon monolayer compression. These results provide a framework for designing F-modified bile acids to mimic or diverge from the natural compounds in vivo.—Kauffman, J. M., R. Pellicciari, and M. C. Carey. **Interfacial properties of most monofluorinated bile acids deviate markedly from the natural congeners: studies with the Langmuir-Pockels surface balance.** *J. Lipid Res.* 2005. 46: 571–581.

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Bile salts are biologically relevant amphiphiles that solubilize biliary and dietary lipids and exert homeostatic control over the body's cholesterol metabolism. They act as natural ligands for the farnesoid X receptor (FXR), a key nuclear transcription factor (1) that regulates many crucial steps in bile salt synthesis, metabolism, and transport. Bile salts are essential for solubilization of sterols (2), thereby promoting their dispersion and secretion within the biliary tract as well as their absorption from the small intestine. The number, position, and orientation of OH substituents on the steroid nucleus of these amphiphilic molecules are functionally expressed in interfacial properties (3, 4) that influence not only detergency and dispersion properties but also interaction with both transcription factors and transporter proteins (1, 2). These properties can be elucidated using bile acids with undissociated carboxylic acid side chains by means of compression of monomolecular layers at an air (which is mostly hydrophobic N₂)-water interface at constant temperature (3, 4). The generation of surface pressure-molecular area (π -A) isotherms reflects the lateral pressures between molecules as functions of their compression into smaller occupancy areas (5). With respect to the common dihydroxy bile acids, π values are increased with compression until they reach a sharp collapse point, after which further compression leads to the formation of trimolecular layers without additional changes in surface π (4, 6). Because bile acids are not volatile at ambient temperatures, deviation from this behavior represents bulk solubility of the molecules (4, 5). Prior work (3, 4, 6) has established that the air-water interfacial orientation of the steroid nucleus in all common dihydroxy bile acids is parallel to the interface with the 3 α -OH group acting as the principal molecular anchor in the aqueous subphase. Addition of a monohydroxy sterol, such as cholesterol, or a common phospholipid, such as 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), to monomolecular

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layers of common bile acids does not affect this horizontal orientation of the molecules and invariably leads to marked condensation of the mixed monolayer (3, 4).

We demonstrated recently (7) that F substitution for a single hydrogen atom on the steroid nucleus of cholesterol, or multiple F substitutions on cholesterol's side chain, failed to appreciably perturb the surface physical-chemical properties of the sterol (7), confirming bulk work done earlier with these molecules (8). Nonetheless, it is known that F substitution in proximity to functional groups can affect enzymatic processes substantially, as demonstrated by the inhibition of cholesterol 7 α -hydroxylation by fluorination at the C-6 position of the sterol (9). The F-carbon bond is highly stable (10) and has an effective van der Waals radius similar to H in the C-H bond (7, 10). Moreover, such substitution has the potential to be detected at the ultrastructural level in vivo by electron spectroscopic imaging, which localizes individual atoms by using the characteristic energy loss of primary electrons (11–13). In addition, minimal F modification of bile acids may substantially alter their affinity for the FXR, the canonical transcription factor for the dihydroxy and trihydroxy bile acids (1, 14–19). Clearly, potential pharmacological advantages should emerge from the ability to enhance or diminish FXR activity by F-modified bile acids (20).

We used an automated Langmuir-Pockels surface balance (3, 7) to characterize the interfacial properties of four mono

F-substituted dihydroxy bile acids and to determine whether F-substituted bile acids resemble the parent compounds. We compared them with their respective natural dihydroxy analogs, alone and in binary mixtures with a common phospholipid, POPC. Because of the potential for hydrophilic F-substitution to enhance aqueous solubility, we compared two of the F-substituted dihydroxy bile acids with natural trihydroxy bile acids containing isologous OH orientations and positions. For this purpose, we chose 3 α ,6 β ,7 α -trihydroxycholeanoic acid [α -muricholic acid (α -MCA)] and 3 α ,6 α ,7 β -trihydroxycholeanoic acid [ω -muricholic acid (ω -MCA)], which are predicted a priori to form unstable monolayers at the air-water interface (4). We found that three of the four mono F-substituted bile acids deviate markedly from the parent analogs because of increased hydrophilicity reflected in interfacial properties similar to those of the trihydroxy α - and ω -MCAs. Nonetheless, the interfacial properties of all monofluorinated bile acids are similar to those of the native homologs in their ability to condense mixed monolayers with POPC.

EXPERIMENTAL PROCEDURES

Materials

Monofluorinated bile acids, 7 α -fluoromurocholic acid (FMCA), 7 α -fluorohyodeoxycholic acid (FHDCa), and 6 α -fluorochole-

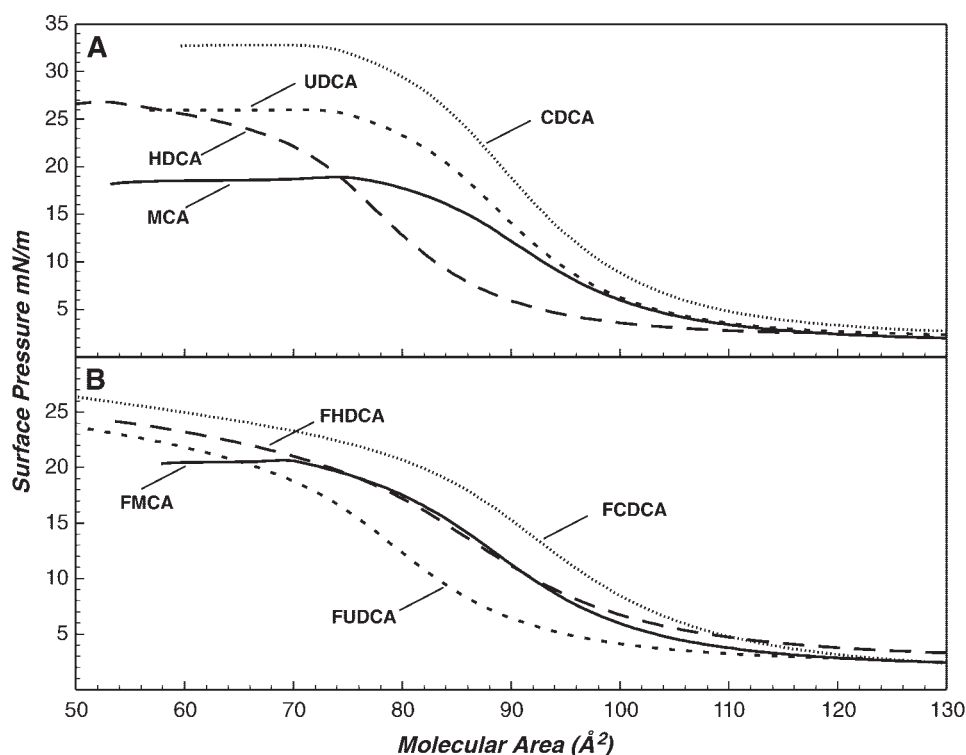


Fig. 1. A: Surface pressure-molecular area (π -A) isotherms at which π is plotted against A for spread monomolecular layers of murocholic acid (MCA), hyodeoxycholic acid (HDCA), ursodeoxycholic acid (UDCA), and chenodeoxycholic acid (CDCA) at $21 \pm 0.5^\circ\text{C}$ on a 5 M NaCl subphase at pH 3. B: π -A isotherms for 7 α -fluoromurocholic acid (FMCA), 7 α -fluorohyodeoxycholic acid (FHDCa), 6 α -fluoroursodeoxycholic acid (FUDCA), and 6 α -fluorocholedeoxycholic acid (FCDCA) under the same experimental conditions. The π -A isotherms of CDCA, UDCA, MCA, and FMCA exhibit sharp collapse points, whereas those of HDCA, FHDCa, FCDCA, and FUDCA show second-order phase transitions, as continuous increases in π accompany all monolayer compressions.

oxycholic acid (FCDC), were synthesized as detailed elsewhere (21). 6 α -Fluoroursodeoxycholic acid (FUDCA) was generously provided by Novartis Pharmaceuticals, Inc. (East Hanover, NJ). Highest purity dihydroxy bile acids, 3 α ,6 β -dihydroxycholanoic acid (MCA), its 6 α -OH epimer, 3 α ,6 α -dihydroxycholanoic acid [hyodeoxycholic acid (HDCA)], 3 α ,7 β -dihydroxycholanoic acid [ursodeoxycholic acid (UDCA)], and its 7-OH epimer, 3 α ,7 α -dihydroxycholanoic acid [chenodeoxycholic acid (CDCA)], were purchased from Sigma Chemical Co. (St. Louis, MO). Highly purified α -MCA and ω -MCA were generous gifts from Tokyo-Mitsubishi Co., Ltd. (Tokyo, Japan). Bile acid purities were >99% as assessed by TLC on silica plates (Fisher Scientific, Pittsburgh, PA) using butanol-acetic acid-water (10:1:1, v/v) as solvent. Synthetic POPC was purchased from Avanti Polar Lipids (Alabaster, AL) and found to be >99% pure by TLC on silica plates using CHCl₃/methanol/NH₄OH (65:25:4, v/v) as mobile phase. HPLC-grade organic solvents were purchased from Fisher Scientific and shown to be free of surface active impurities by layering 100 μ l onto the subphase and observing no appreciable deviation from zero pressure during film compression. After reverse osmosis, deionized water was distilled and filtered through activated charcoal. Sodium chloride was roasted at >600°C in air for 6–8 h to oxidize and remove organic impurities. Glassware was alkali washed in ethanol/2 M KOH (1:1, v/v), then soaked in 1 M HNO₃, followed by generous rinsing in distilled water.

Spreading solutions

To prepare spreading solutions, stock solutions were composed first in methanol/CHCl₃ (8:2, v/v) at an estimated concentration of 12–15 mg/ml. These solutions were then filtered through an organic solvent-resistant Millex-LCR13 0.5 μ m filter (Millipore, Billerica, MA). Dry weights were measured using qua-

druplicate 100 μ l volumes, with evaporation of the solvent under reduced pressure in a desiccator at 35°C for 1 h. Spreading solutions for all bile acids and POPC were prepared with 3:1 hexane-ethanol to yield final concentrations of 0.4–0.7 mM. To prevent chemical degradation and evaporation, spreading solutions were stored in Teflon-sealed containers under argon at 4°C for periods no longer than 6–8 weeks.

Measurements of surface pressures and molecular areas

π -A isotherms were obtained using an automated Langmuir-Pockels surface balance in a configuration as described previously (3, 7) consisting of an 8.5 \times 36.5 cm Teflon-lined trough and computer-controlled barriers (KSV Instruments, Ltd., Helsinki, Finland). Minor modifications to the procedures were as follows. Monomolecular layers of the natural dihydroxy bile acids and corresponding F analogs, alone and in binary mixtures with POPC, were spread using 100 μ l glass syringes (Hamilton Co., Reno, NV) on a subphase of 5 M NaCl at pH 3 [in contrast to pH 2 used by Fahey, Carey, and Donovan (3)], with temperature of 21 \pm 0.5°C. The natural trihydroxy bile acids (α -MCA and ω -MCA) were studied as pure monomolecular layers only. All spreading solvents were allowed to evaporate for 5 min before surface compression of the monolayers. π -A values at “lift-offs” and collapse points (3, 22) were extrapolated from intersections of tangents drawn on each side of the curves where the isotherms deviated in a first-order manner. Between three and five individual isotherms were run for each bile acid and binary mixtures with POPC, with lift-off areas reproducible to within 1%. Representative isotherms are plotted in the figures. To estimate bulk solubility of fluorinated bile acids, FUDCA was studied as a test compound, with UDCA as a control. As in the other experiments, pure monolayers of these bile acids were spread individu-

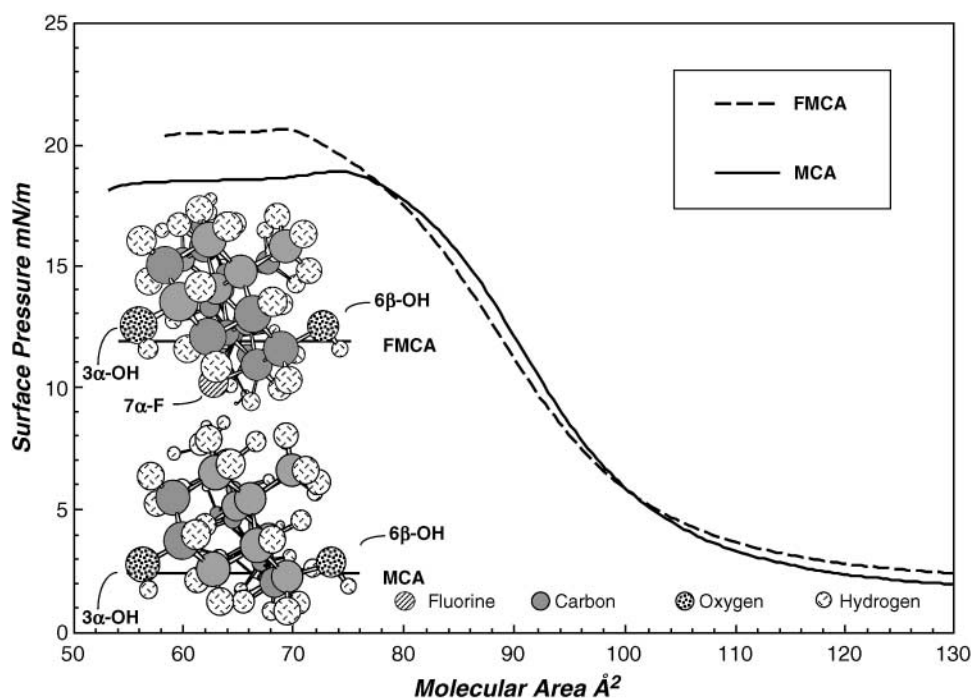


Fig. 2. π -A isotherms of FMCA and MCA with cross-sectional depictions of their respective ball-and-stick molecular models shown at the air-water interface (horizontal solid lines). The isotherms of both FMCA and MCA produce true collapse points at 21.5 and 19 mN/m, respectively, corresponding to molecular areas of 70 and 75 \AA^2 . However, in contrast to other fluorinated bile acids and relative to the parent molecule, the isotherm of FMCA displays a higher collapse pressure. Of note is that the 7 α -F atom and the 6 β -OH group in FMCA are not in close proximity, and both project isolaterally into the subphase.

ally on identical (5 M NaCl, pH 3) subphases at $21 \pm 0.5^\circ\text{C}$. After evaporation of the solvent, the surface area of the trough was decreased until π reached 10 mN/m, and the rate of diminution in A values at this constant π value was recorded over 5 min.

Software for molecular modeling

ChemDraw Pro and Chem3D Standard (Cambridge Scientific, Cambridge, MA) were used to develop two- and three-dimensional models of the natural bile acids and their F derivatives. Energy minimization of three-dimensional models was performed whenever appropriate. The ball-and-stick molecular models are drawn in the figures at hypothetical air-water interfaces with areas corresponding to projections of the molecules in a two-dimensional gas.

RESULTS

Pure bile acid monolayers

Figure 1A shows the π -A isotherms of the nonfluorinated natural bile acids MCA, HDCA, UDCA, and CDCA. All isotherms demonstrate similar characteristics, exhibiting low pressures at 2 and 3 mN/m for surface areas greater than 120 \AA^2 . With monolayer compression, all isotherms lift-off at molecular areas between 100 and 120 \AA^2 , after which further compression results in progressive increases in π values. These findings are consistent with the molecules lying flat at the air-water interface (3, 4), with the OH groups of the steroid nucleus and the carboxylic acid groups anchored in the subphase (4). With continued compression, collapse points occur at π values of 19,

27, 26, and 33 mN/m for MCA, HDCA, UDCA, and CDCA, respectively.² With compression beyond the collapse points, the surface π values remain stable, except for a slight downward decline in the case of HDCA, the only natural bile acid studied with a 6α (equatorial) -OH function. It is apparent that this isotherm exhibits a second-order phase transition between 65 and 75 \AA^2 , suggestive of π -induced interfacial tilting of the molecules during compression.

Figure 1B depicts the π -A isotherms of the monofluorinated bile acids FMCA, FHDCA, FUDCA, and FCDCA. As found with their natural congeners (Fig. 1A), all isotherms display low interfacial π values at A values greater than 120 \AA^2 . Upon compression, the characteristics of the isotherms become distinctly different from those of the native homologs. With the sole exception of FMCA, none of the F-substituted bile acids demonstrate distinct first-order collapse points. A true collapse point (FMCA) and apparent collapse points (FHDCA, FUDCA, and FCDCA) in-

² Previously reported π values at film collapse for spread monolayers of UDCA and CDCA from this laboratory (3, 23) differ somewhat from one another as well as from the collapse π values in the current work. This is most likely attributable to differing conditions (pH, temperature, composition of the subphase, and sizes of the surface balance troughs) used. Whereas the π values at collapse vary among studies, their rank order at the collapse points, as well as A values at lift-off, remain consistent among these reports and are in general agreement with the present work.

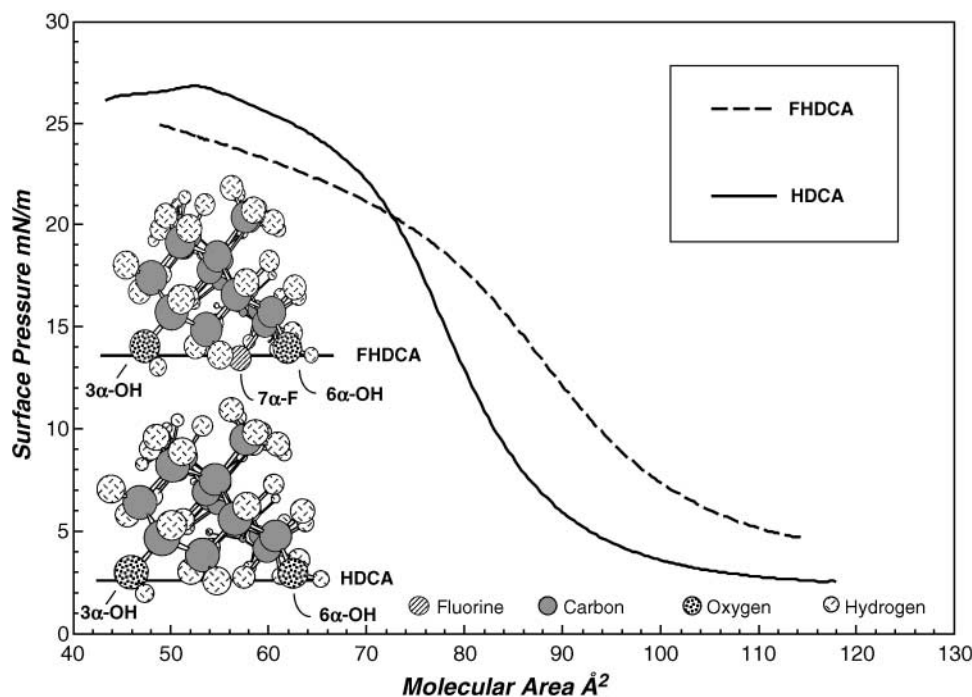


Fig. 3. π -A isotherms of FHDCA and HDCA with cross-sections of the corresponding molecular models at the air-water interface. Both FHDCA and HDCA isotherms display second-order phase transitions, FHDCA between 75 and 85 \AA^2 and HDCA between 65 and 75 \AA^2 , with no clear collapse point in the case of FHDCA. The sharp collapse point in the HDCA isotherm occurs at a surface π of 27 mN/m and an A of 55 \AA^2 . The molecular models show that the 7α -F (axial) and 6α -OH (equatorial) groups are in moderately close proximity.

terpolate to π values of 21.5, 21, 20, and 22 mN/m, respectively. The latter three collapse points are pseudo-order because the surface π values continue to increase with continued compression of the monolayers. In general, in the case of F-substituted bile acids, the π and A values at collapse or second-order phase transitions are lower than those of the natural dihydroxy bile acids, with the exception of FMCA. In this case, the π value is higher at monolayer collapse and the A value is smaller than the corresponding values for the natural congener (Fig. 1A, B). Nonetheless, the same rank order of π and A (collapse/pseudocollapse) values is generally followed for F-substituted and natural bile acids.

Figure 2 displays π -A isotherms and three-dimensional molecular models of MCA and FMCA at the air-water interface. Inspection of the molecular models illustrates that the 7α -F atom does not lie in close proximity to the 6β -OH group and, moreover, is remote from the 3α -OH group. The isotherms exhibit striking similarities at molecular A values greater than 80 \AA^2 . With progressive compression, there is first-order collapse of the monolayer in both cases, but with a smaller A and larger π for FMCA. The molecular models show that the positioning of the 3α - and 6β -hydroxyl groups at the interface allows the 7α -F atom to project into the subphase, along with several CH_2 groups of the steroidal A and B rings. This configuration does not alter the lift-off, slope, or collapse π or A values of the isotherms appreciably (note the near identity of

curves in Fig. 2) and suggests that the interfacial orientations of 3α - and 6β -OH functions are unaffected by F substitution at the 7α position. Because the collapse point of FMCA occurs at a higher π than is the case with MCA, these data suggest that the nonvicinal hydrophilic F actually stabilizes the bile acid in the interface, possibly because of aqueous immersion of several hydrophobic portions of the steroid nucleus (Fig. 2, models).

Figure 3 depicts π -A isotherms and displays molecular models for HDCA and FHDCA. The isotherm of the natural HDCA increases steeply with continued compression and shows a second-order phase transition between 65 and 75 \AA^2 and an apparent collapse point at $\sim 55 \text{ \AA}^2$. In contrast, with FHDCA there is lift-off at a greater A value; however, no distinct collapse point is evident in the isotherm, although a second-order phase transition occurs between 75 and 85 \AA^2 . At the second-order phase transitions, the π value for FHDCA is smaller and the A value is larger than the corresponding values for HDCA. Inspection of the molecular models shows that the 7α -F atom of FHDCA is located at the air-water interface in close proximity to the 6α -OH group (Fig. 3, models).

Figure 4 plots π -A isotherms of UDCA and FUDCA, which are appreciably different from each other, and also shows the molecular models of the bile acids. The isotherm of UDCA lifts off at a higher A value than FUDCA and demonstrates a collapse at a similar A value ($\sim 75 \text{ \AA}^2$) to the mid point of the second-order phase transition in

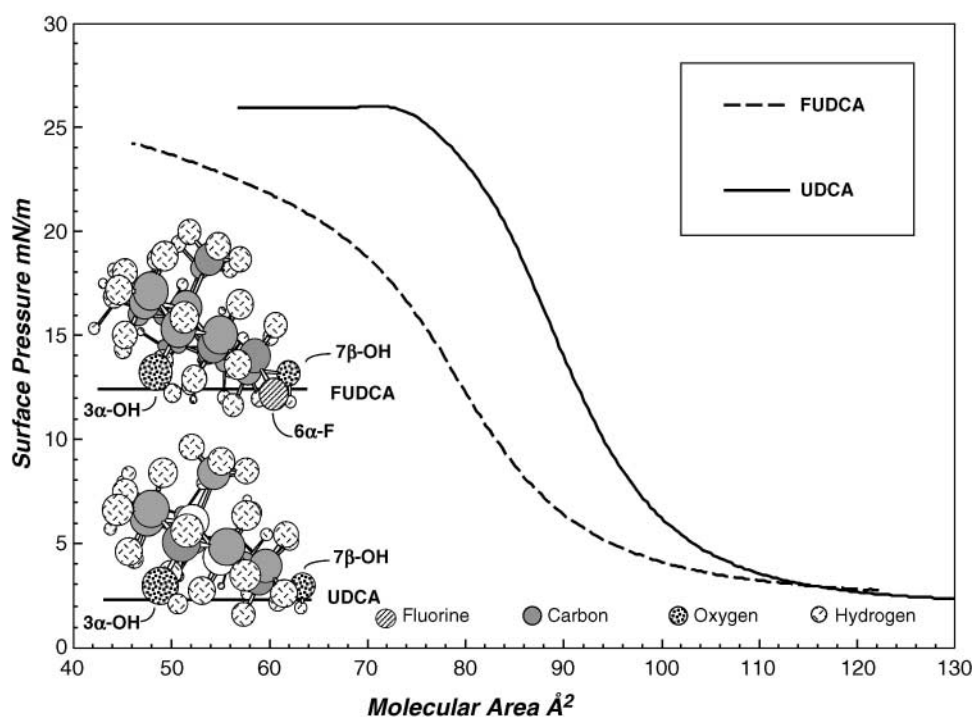


Fig. 4. π -A isotherms of FUDCA and UDCA and cross-sections of molecular models for each at the air-water interface. The FUDCA isotherm is shifted markedly to smaller molecular areas compared with the isotherm of UDCA, suggesting bulk solubility of the FUDCA molecules (proven experimentally in this work; see Results). The collapse point for UDCA interpolates to a surface π of 26 mN/m and an A of 76 \AA^2 . As suggested by the molecular model, the 6α -F atom of FUDCA rests at the interface and lies in close proximity to the equatorial 7β -OH group.

the FUDCA isotherm. Inspection of the molecular models shows that the 6α -F atom in FUDCA lies at the interface in close proximity to the equatorial 7β -OH group. The marked shift to the left in the FUDCA isotherm suggests appreciable bulk solubility of the molecules beginning with even the smallest compressions. This hypothesis was tested empirically using changes in A values for FUDCA and UDCA monolayers at constant π when spread individually on an identical subphase (see Experimental Procedures). With compression to a constant π of 10 mN/m, the isotherm of UDCA revealed no decline in A, whereas the A value of FUDCA diminished by 8.5% with the passage of time (5 min). Based on this surface loss, an arithmetic calculation indicates that at this π value, 3% of FUDCA molecules leave the interface every 5 min (22).

Figure 5 plots π -A isotherms and molecular models for CDCA and FCDCA. The FCDCA isotherm is shifted to the left, with a more gradual increase in π after lift-off compared with the natural CDCA. After collapse at an A value of $\sim 73 \text{ \AA}^2$, further compression of the CDCA monolayer results in a constant π value with diminishing A values. In contrast, in the case of FCDCA, there is a second-order phase transition at an A value of $70\text{--}90 \text{ \AA}^2$, after which there is a gradual and continuous increase in π . The molecular model shows that the 6α -F atom in FCDCA is equatorial and the 7α -OH group is axially oriented.

To test the hypothesis that most F substitutions confer hydrophilic properties on a bile acid molecule, we compared the interfacial properties of F-substituted bile acids to the natural trihydroxy bile acids that were closest with

regard to number, position, and orientation of the three polar OH substitutions on the bile acid's steroid nucleus (α -MCA and ω -MCA). Based on an analogy with cholic acid (4), these were predicted a priori to be soluble in the 5 M NaCl, pH 3 bulk phase upon monolayer compression. The OH functions of α -MCA (3α -OH, 6β -OH, 7α -OH) and ω -MCA (3α -OH, 6α -OH, 7β -OH) are substituents in isologous positions and orientations to the F atoms and OH functions of FMCA (3α -OH, 6β -OH, 7α -F) and FUDCA (3α -OH, 6α -F, 7β -OH). **Figure 6** shows the π -A isotherms of α -MCA and ω -MCA along with their fluorinated analogs FMCA and FUDCA. Both α -MCA and ω -MCA as well as FUDCA display second-order phase transitions. Because a true collapse point is not evident during monolayer compression, it is apparent that these trihydroxy bile acids exhibit interfacial instability and do so even more than cholic acid (3, 4). This unstable interfacial behavior results from amplification of the hydrophilic properties of a steroid molecule by the third OH group, particularly when α - and β -OH substituents are vicinal on the steroid nucleus (4). Compared with ω -MCA, the π -A isotherm of FUDCA is shifted to smaller molecular areas, which is consistent with the experimentally proven loss of molecules into the subphase. Therefore, FUDCA is more hydrophilic than ω -MCA. Isotherms for FMCA and α -MCA show similar lift-off areas, but with continued compression, the isotherms deviate from each other in that FMCA undergoes a sharp collapse at 21 mN/m, whereas α -MCA displays a progressive increase in π beyond a pseudo-order collapse point.

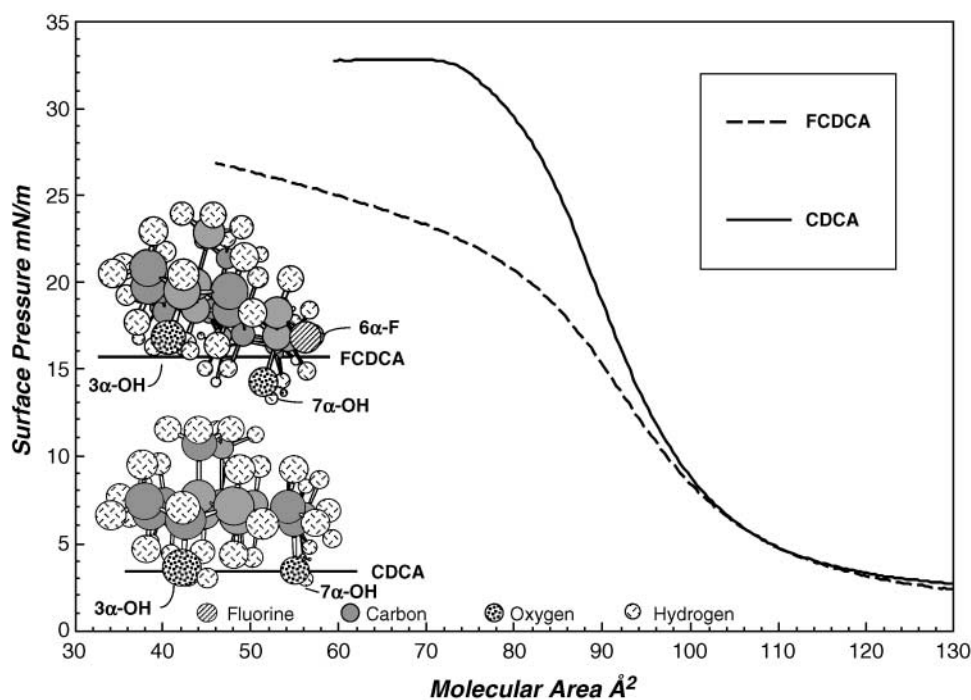


Fig. 5. π -A isotherms of FCDCA and CDCA and corresponding cross-sectional views of their molecular models at an air-water interface. The FCDCA isotherm is shifted to the left of that for CDCA, displaying a more gradual increase in surface π and a pseudocollapse region corresponding to a molecular A of $\sim 80 \text{ \AA}^2$. The isotherm of CDCA demonstrates a well-defined collapse point at an A of 75 \AA^2 . Of note in the molecular model of FCDCA is the proximity of the 6α -F atom to the 7α -OH group.

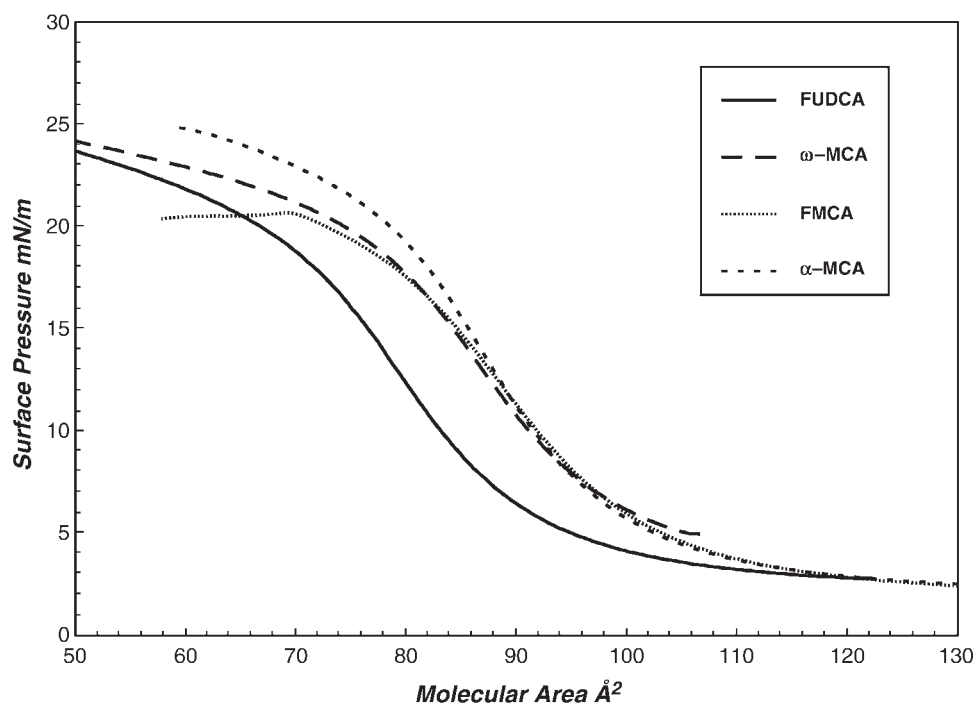


Fig. 6. π -A isotherms of FUDCA, ω -MCA, FMCA, and α -MCA. FUDCA and ω -MCA are comparable binary pairs in that both possess 3α -OH and 7β -OH functions and differ only in their F or OH substituent, respectively, at the 6α position. Similarly, FMCA and α -MCA are comparable binary pairs with 3α -OH and 6β -OH functions and differ only in their F or OH substitution, respectively, at the 7α position. Only the isotherm of FMCA displays a well-defined collapse point. The isotherm of FUDCA is shifted to the left of ω -MCA, with lift-off and pseudocollapse points that occur at lower molecular A values for the natural analog, indicative of its greater bulk solubility. In contrast, FMCA has no appreciable bulk solubility, as indicated by its sharp collapse point, in contrast to its trihydroxy α -MCA analog.

Bile acid-POPC mixed monolayers

Figure 7 shows representative π -A isotherms for binary mixtures of MCA with POPC (A) and FMCA with POPC (B), covering the range of molar compositions from 0% to 100% bile acid. π decreases markedly when POPC is added to bile acid monolayers in the expanded state. However, with continued compression, POPC increases π in the mixed monolayers. Moreover, the collapse points are progressively eliminated with increasing mole percent POPC (Fig. 7A, B).

Figure 8 plots additivity curves (3, 4, 7, 22) for binary mixtures of POPC with MCA and FMCA (A), POPC with HDCA and FHDCA (B), POPC with UDCA and FUDCA (C), and POPC with CDCA and FCDCA (D). The curves represent bile acid-to-POPC molar stoichiometries in progressively increasing bile acid increments, all at a constant π of 10 mN/m. Dotted lines represent ideal additivity behavior for the A values of POPC and the A values of the respective bile acid molecules. Negative deviations from ideality represent smaller molecular areas (i.e., condensation) compared with those predicted by the sum of the fractional contributions of POPC and the bile acid. Figure 8A demonstrates that, for all mixtures of MCA or FMCA with POPC, similar degrees of negative deviation occur, except at 15 mol%, at which value FMCA displays somewhat less deviation than does MCA. However, maximal negative deviations for both MCA or FMCA and POPC are reached from 30 to 70 mol% bile acid. Figure 8B reveals that HDCA and FHDCA

produce substantially different surface areas of 83 and 93 \AA^2 , respectively. In all mixtures with POPC, both HDCA and FHDCA show negative deviations from ideality, with minimal values occurring at ~ 50 mol% bile acid. In the case of the 85 mol% mixture of FHDCA with POPC, the curve shows a modest positive deviation, suggestive of monolayer expansion. This contrasts with the condensation induced by HDCA at 85 mol% in the mixed POPC-bile acid monolayer. Figure 8C shows that UDCA manifests a larger surface area (94 \AA^2) than FUDCA (83 \AA^2) at the π value used (10 mN/m). However, with all binary mixtures of UDCA or FUDCA with POPC, the curves deviate negatively from ideality, illustrating maximal deviations at 30 mol% bile acid. FUDCA demonstrates a modest negative deviation at 85 mol% bile acid, similar to the isomolar mixture of FMCA and POPC (Fig. 8A). Figure 8D shows that for all molar mixtures, negative deviations from ideality occur for CDCA or FCDCA with POPC except for the 85 mol% CDCA composition. For FCDCA and POPC, maximal negative deviation occurs at 50 mol%, whereas for CDCA and POPC, the corresponding value occurs at ~ 30 mol% bile acid.

DISCUSSION

The fundamental relevance of the interfacial properties of amphiphiles to their interactions with membranes, with

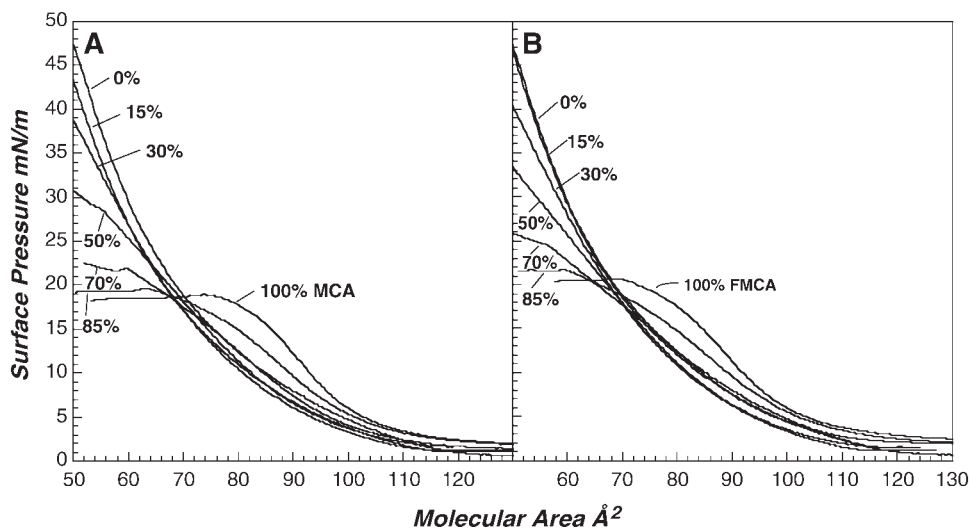


Fig. 7. π -A isotherms of binary mixtures of MCA (A) and FMCA (B) with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) as functions of mole percent bile acid, as indicated on each curve. The isotherms for both bile acids are similar, except for the FMCA isotherm at the 15% admixture, which closely parallels the isotherm for pure POPC.

transporters, and with otherwise insoluble lipids is well known. Of particular importance are the interactions of bile acids with nuclear receptors such as FXR (20). Our studies demonstrate the critical role of the location of a single F substitution on the steroid nucleus of common di-

hydroxy bile acids in influencing their interfacial properties. When placed in close proximity to an OH group, the hydrophilic effect of F is uniformly amplified, as illustrated by FUDCA (Fig. 4). In this derivative, the 6 α -F and 7 β -OH groups lie in a *cis* equatorial projection on the ste-

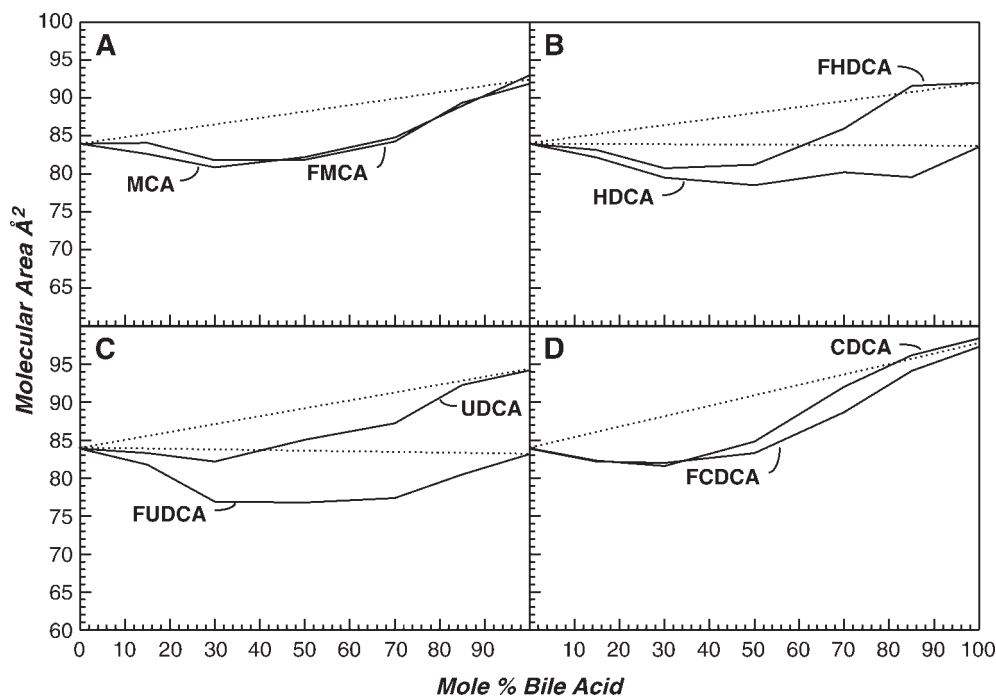


Fig. 8. Area additivity curves for POPC in binary mixtures with FMCA and MCA (A), with FHDCA and HDCA (B), with FUDCA and UDCA (C), and with FCDCA and CDCA (D). Molecular area is plotted against mole percent bile acid in each mixture, for a constant surface pressure at 10 mN/m. Dashed lines represent ideal additivity behavior of the molecular areas of POPC with each bile acid. Curves for MCA and FMCA are essentially identical, and both condense POPC maximally at \sim 50 mol%. FHDCA displays no condensation at 85 mol%, but HDCA does. FUDCA induces greater condensation of POPC than UDCA, especially at 85 mol% bile acid, whereas UDCA induces minimal condensation at this composition. CDCA and FCDCA condense POPC monolayers at all concentrations <85 mol% bile acid.

roid nucleus. In contrast, there appears to be much less amplification in the case of other F-bile acids and none in the case of FMCA (Fig. 2). Moreover, FUDCA demonstrates progressive solubility in the subphase (see Results), further supporting the augmented hydrophilic effect of an F substitution vicinal to an OH function. Our results also display marked contrast in the interfacial behaviors of FUDCA and ω -MCA, suggesting that there is greater hydrophilicity of FUDCA compared with the isologous trihydroxy bile acid. However, this was not as clearly marked in the comparison of FMCA and α -MCA (Fig. 6). Moreover, such data invariably predict that, when ionized, the critical micellar concentrations of fluorinated dihydroxy bile salts should be larger and partition coefficients should be smaller than for trihydroxy bile salts.

Fluorination of biologically active molecules alters their potential to increase or decrease most pharmacological effects. In general, anticancer, antiviral, and anti-inflammatory agents have all been successfully modified by F substitution to produce more active compounds (24, 25). In some cases, fluorination affects the surface chemistry of the molecules, whereas in other cases, it provides no appreciable effect. For example, the perfluorination of eicosane, an aliphatic hydrocarbon without a polar group, results in ordering of the fluorinated lipid at the air-water interface (26). Mono F substitution in the steroid nucleus and even hepta F substitution of cholesterol's side chain resulted in interfacial properties of the molecules that were virtually indistinguishable from cholesterol itself (7, 8). This contrasts appreciably with data in the present work, which show that, in most cases, a single F substitution in the steroid nucleus of bile acids appears to influence the surface physical chemistry of the molecules markedly, especially by augmenting hydrophilicity. These oxidative derivatives of cholesterol generally subtend two or three OH functions on the α side of the steroid nucleus and, as a result, allow them to behave as planar amphiphiles with low critical micellar concentrations when ionized, thereby promoting lipid solubility in the biliary tree and alimentary tract (2). Most notably, the dihydroxy bile acids studied here all possess a 3α -OH group but subtend a second OH group at either the C6 or C7 position in α or β orientation. In addition, the F-substituted dihydroxy bile acids contain the F atom at the 6α position in the case of the 7-OH bile acids UDCA and CDCA and at the 7α position in the case of the 6-OH bile acids MCA and HDCA. Hydrophilicity is augmented as a result of these vicinal polar interactions, as displayed in the case of most F derivatives studied.

The vicinal effect of 6α -F substitution on the stability of UDCA toward enzymatic dehydroxylation (27) is known to be augmented in FUDCA because it is more resistant to bacterial 7-dehydroxylation than is UDCA. Here, we show that the effect of F substitution on a bile acid's surface chemistry is greatly dependent upon the proximity of F and OH groups. The 6β -OH and 7α -F projections of FMCA (Fig. 2) result in functional groups that exhibit a poor capacity to potentiate the hydrophilic properties of one another. This is likely attributable to the projection of dis-

similar functional groups toward opposite sides of the steroid nucleus, as displayed by the molecular models (Fig. 2). The π -A isotherms, therefore, are very similar, reaching collapse points at similar molecular A values (Fig. 2). The higher surface π at collapse of FMCA is consistent with the unamplified hydrophilic nature of F, which results in a more stable monolayer. The HDCA isotherm (Fig. 3) is distinct from those of the other natural bile acids in that it demonstrates a second-order phase transition between 65 and 75 \AA^2 , with a collapse point at $\sim 55 \text{\AA}^2$. This anomalous behavior, most likely attributable to tilting of the molecules in the interface, is exaggerated in the isotherm of FHDCA, in which no distinct collapse point occurs and a broad second-order phase transition is evident between 75 and 85 \AA^2 . In addition, FHDCA is the only F-substituted bile acid whose π -A isotherm is shifted to the right of the isotherm of the natural bile acid at low π but to smaller A values than HDCA at high π . Upon examining the molecular models (Fig. 3), the respective equatorial and axial projections of the 6α - (OH) and 7α - (F) functional groups place them in close proximity on the steroid nucleus, permitting an amplification of their hydrophilic properties (4) when constrained in the monolayer (Fig. 3). As displayed in the molecular models of FUDCA and UDCA (Fig. 4), the F and OH groups are in close proximity, which results in an exaggerated hydrophilic effect. The π -A isotherm of FUDCA is shifted markedly to the left at all π values, indicative of bulk solubility of the molecules ($\sim 3\%$ loss in 5 min), even at a low π value (see Results). Figure 5 displays the molecular models for CDCA and FCDCA, wherein the 6α -F and 7α -OH groups project from the steroid nucleus in close proximity. In contrast, in FUDCA and UDCA (Fig. 4), the F and OH functional groups in the 6α - (F) and 7β - (OH) positions are reversed in orientation on the molecular models. Nonetheless, the overall hydrophilic effects between CDCA and UDCA and their fluorinated derivatives are similar, with a second-order phase transition occurring in FCDCA and FUDCA (Figs. 4, 5).

There are three natural MCA isomers, but only the more common β -MCA (3α -OH, 6β -OH, 7β -OH) has been studied at an air-water interface in past work (28). That experiment provided evidence of bulk solubility of β -MCA molecules in acidic subphases upon monolayer compression (4, 28). The trihydroxy isomers studied in the present work, α -MCA and ω -MCA, possess an OH function at isologous orientations and positions on the bile acid nucleus to the OH and F functions in FMCA and FUDCA. Comparison of ω -MCA and FUDCA isotherms (Fig. 6) suggest that the 6α -F substitution leads to greater bulk solubility than that produced by the 6α -OH group because the FUDCA isotherm is shifted to smaller molecular areas than ω -MCA at all π values. The FMCA and α -MCA bile acids are likewise structurally similar in that both subtend 3α - and 6β -OH groups, but in contrast to the 7α -OH group of α -MCA, FMCA contains a 7α -F group. The isotherms of both of these bile acids display lift-offs at similar areas, but with respect to the monofluorinated bile acids studied, FMCA is the only one that behaves as an insoluble

ble amphiphile (4), producing a stable monomolecular layer with a sharp collapse point at 70 Å² (Fig. 2). The greater hydrophilicity of α-MCA (showing no collapse point) compared with FMCA is anomalous in that it is only apparent at high but not low π values. However, it may mean less steric hindrance for hydration of the equatorial 6β-OH function in α-MCA than would occur in the case of FMCA. Inspection of the molecular models reveals that, in the latter, hydrocarbon moieties of the A/B rings are exposed unfavorably to the subphase (Fig. 2).

In binary mixtures with POPC, all F-substituted bile acids generally behave in a manner similar to each other and to their native congeners, resulting in strong condensation of the monolayers, which is maximal in most cases at either 30 or 50 mol% bile acids. Isolated exceptions are evident at the highest concentration of FHDCA, UDCA, and CDCA (Fig. 8). This suggests that the presence of the F atom does not appreciably perturb the bile acid ordering of the acyl chains of POPC (4). As anticipated by similar isotherms for pure FMCA and MCA, all mixtures of FMCA or MCA with POPC result in highly similar molecular areas at low surface pressure (10 mN/m).

Pathobiologic implications

The pharmacologic utility and possible use of fluorinated bile acids as structural biologic labeling agents are yet to be fully explored. Clearly, their potential application as tracer molecules in electron spectroscopic imaging (13) is promising, and as suggested by the present results, it may be appropriate to eschew the unstable interfacial properties of FCDCA, FUDCA, and FHDCA and use FMCA or an analog for in vivo spectroscopic imaging studies. Because bile acids are canonical ligands for FXR (14–20), their activity will depend not only upon their transport into cells but also upon their ability to bind to and activate this nuclear receptor (20). Therefore, their recognition by transport proteins and enzymatic systems as (pseudo) endobiotics rather than xenobiotics will be a significant advantage. The hydrophilic-hydrophobic balance of an F-modified bile acid, a property that is reflected in the surface behavior of its spread monomolecular layer at the air-water interface, is therefore of primary relevance in testing its potential to act as putative nuclear receptor ligand. One of us (R.P.) reported recently that acyl substitution at the 6α position of CDCA produces a potent and selective FXR agonist (29). As was the case with fluorinated steroid hormone derivatives (24), F-substituted bile acids may be resistant to degradation pathways apart from that of bacterial 7-dehydroxylation (27). These molecules therefore represent agents of potential pharmacological importance with FXR as their primary target. We conclude that, in designing such semisynthetic molecules, the F substituent should not be vicinal to an OH function for optimal interfacial stability and presumably for protein binding. In summary, our work demonstrates that monofluorination of the steroid nucleus of common bile acids has the potential to markedly alter the hydrophilic-hydrophobic character of these molecules, depending upon the vicinal or distal placement of the F atom to an OH function.

Such information should aid in the design, synthesis, and screening of fluorinated bile acids to more faithfully mimic or deviate from the physical-chemical behavior of the natural congeners (29). ■

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REFERENCES

1. Chiang, J. Y. 2003. Bile acid regulation of hepatic physiology. III. Bile acids and nuclear receptors. *Am. J. Physiol.* **284**: G349–G356.
2. Carey, M. C., and W. C. Duane. 1994. Enterohepatic circulation. In *The Liver: Biology and Pathobiology*, 3rd edition. I. M. Arias, J. L. Boyer, N. Fausto, W. B. Jakoby, D. Schachter, and D. A. Shafritz, editors. Raven Press, New York. 719–767.
3. Fahey, D. A., M. C. Carey, and J. M. Donovan. 1995. Bile acid/phosphatidylcholine interactions in mixed monomolecular layers: differences in condensation effects but not interfacial orientation between hydrophobic and hydrophilic bile acid species. *Biochemistry*. **34**: 10886–10897.
4. Small, D. M. 1971. The physical chemistry of cholanic acids. In *The Bile Acids*. Vol. 1. P. P. Nair and D. Kritchevsky, editors. Plenum Press, New York. 249–356.
5. Gaines, G. L. 1966. *Insoluble Monolayers at Liquid-Gas Interfaces*. Wiley Interscience, New York.
6. Carey, M. C. 1985. Physical chemical properties of bile acids and their salts. In *New Comprehensive Biochemistry*. Vol. 12. Sterols and Bile Acids. H. Danielsson and J. Sjövall, editors. Elsevier, Amsterdam. 345–403.
7. Kauffman, J. M., P. W. Westerman, and M. C. Carey. 2000. Fluorocholesterols, in contrast to hydroxycholesterols, exhibit interfacial properties similar to cholesterol. *J. Lipid Res.* **41**: 991–1003.
8. Reid, D. G., L. K. MacLachlan, K. E. Suckling, A. Gee, S. Cresswell, and C. J. Suckling. 1991. A fluorine-19 and deuterium NMR study of two novel fluorosterols and their properties in model membranes and high density lipoprotein. *Chem. Phys. Lipids*. **58**: 175–181.
9. Harte, R. A., S. J. Yeaman, J. McElhinney, C. J. Suckling, B. Jackson, and K. E. Suckling. 1996. Effects of novel synthetic sterol probes on enzymes of cholesterol metabolism in cell-free and cellular systems. *Chem. Phys. Lipids*. **83**: 45–59.
10. Bondi, A. 1964. van der Waals volumes and radii. *J. Phys. Chem.* **68**: 441–451.
11. Costa, J. L., D. C. Joy, D. M. Maher, K. L. Kirk, and S. W. Hui. 1978. Fluorinated molecule as a tracer: difluoroserotonin in human platelets mapped by electron energy-loss spectroscopy. *Science*. **200**: 537–539.
12. Crawford, J. M., S. Barnes, R. C. Stearns, C. L. Hastings, and J. J. Godleski. 1994. Ultrastructural localization of a fluorinated bile salt in hepatocytes. *Lab. Invest.* **71**: 42–51.
13. Leapman, R. D., and N. W. Rizzo. 1999. Towards single atom analysis of biological structures. *Ultramicroscopy*. **78**: 251–268.
14. Makishima, M., A. Y. Okamoto, J. J. Repa, H. Tu, R. M. Learned, A. Luk, M. V. Hull, K. D. Lustig, D. J. Mangelsdorf, and B. Shan. 1999. Identification of a nuclear receptor for bile acids. *Science*. **284**: 1362–1365.
15. Parks, D. J., S. G. Blanchard, R. K. Bledsoe, G. Chandra, T. G. Conslor, S. A. Kliewer, J. B. Stimmel, T. M. Willson, A. M. Zavacki, D. D. Moore, and J. M. Lehmann. 1999. Bile acids: natural ligands for an orphan nuclear receptor. *Science*. **284**: 1365–1368.
16. Wang, H., J. Chen, K. Hollister, L. C. Sowers, and B. M. Forman. 1999. Endogenous bile acids are ligands for the nuclear receptor FXR/BAR. *Mol. Cell*. **3**: 543–553.
17. Redinger, R. N. 2003. The role of the enterohepatic circulation of bile salts and nuclear hormone receptors in the regulation of cho-

- lesterol homeostasis: bile salts as ligands for nuclear hormone receptors. *Can. J. Gastroenterol.* **17**: 265–271.
18. Cui, J., L. Huang, A. Zhao, J. L. Lew, J. Yu, S. Sahoo, P. T. Meinke, I. Royo, F. Pelaez, and S. D. Wright. 2003. Guggulsterone is a farnesoid X receptor antagonist in coactivator association assays but acts to enhance transcription of bile salt export pump. *J. Biol. Chem.* **278**: 10214–10220.
 19. Downes, M., M. A. Verdecia, A. J. Roecker, R. Hughes, J. B. Hogensh, H. R. Kast-Woelbern, M. E. Bowman, J. L. Ferrer, A. M. Anisfeld, P. A. Edwards, J. M. Rosenfeld, J. G. Alvarez, J. P. Noel, K. C. Nicolaou, and R. M. Evans. 2003. A chemical, genetic, and structural analysis of the nuclear bile acid receptor FXR. *Mol. Cell.* **11**: 1079–1092.
 20. Mi, L. Z., S. Devarakonda, J. M. Harp, Q. Han, R. Pellicciari, T. M. Willson, S. Khorasanizadeh, and F. Rastinejad. 2003. Structural basis for bile acid binding and activation of the nuclear receptor FXR. *Mol. Cell.* **11**: 1093–1100.
 21. Pellicciari, R., A. Roda, and G. Frigerio. 1991 and 1992. Fluorinated bile acid derivatives, processes for the preparation thereof and pharmaceutical compositions containing them. US Patents 5,061,701 and 5,175,320.
 22. Leonard, M. R., M. A. Bogle, M. C. Carey, and J. M. Donovan. 2000. Spread monomolecular films of monohydroxy bile acids and their salts: influence of hydroxyl position, bulk pH and association with phosphatidylcholine. *Biochemistry.* **39**: 16064–16074.
 23. Carey, M. C., J.-C. Montet, M. C. Phillips, M. J. Armstrong, and N. A. Mazer. 1981. Thermodynamic and molecular basis for dissimilar cholesterol-solubilizing capacities by micellar solutions of bile salts: cases of sodium chenodeoxycholate and sodium ursodeoxycholate and their glycine and taurine conjugates. *Biochemistry.* **20**: 3637–3648.
 24. Filler, R., and Y. Kobayashi. 1982. Biomedical Aspects of Fluorine Chemistry. Kodansha Scientific Books, Tokyo.
 25. Filler, R. 1986. Biologically-active fluorochemicals. *J. Fluor. Chem.* **33**: 361–375.
 26. Li, M., A. A. Acero, H. Zhengqing, and S. A. Rice. 1994. Formation of an ordered Langmuir monolayer by a non-polar chain molecule. *Nature.* **367**: 151–153.
 27. Roda, A., R. Pellicciari, C. Polimeni, C. Cerre, G. C. Forti, B. Sadeghpour, E. Sapigni, A. M. Gioacchini, and B. Natalini. 1995. Metabolism, pharmacokinetics, and activity of a new 6-fluoro analogue of ursodeoxycholic acid in rats and hamsters. *Gastroenterology.* **108**: 1204–1214.
 28. Shibata, O., H. Miyoshi, S. Nagadome, G. Sugihara, and H. Igimi. 1991. Mixed monolayer properties of bile acids spread on the concentrated sodium chloride solution. *J. Colloid Interface Sci.* **146**: 594–597.
 29. Pellicciari, R., S. Fiorucci, E. Camaioni, C. Clerici, G. Costantino, P. R. Maloney, A. Morelli, D. J. Parks, and T. M. Willson. 2002. 6 α -ethyl-chenodeoxycholic acid (6-ECDCA), a potent and selective FXR agonist endowed with anticholestatic activity. *J. Med. Chem.* **45**: 3569–3572.