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^{1}H NMR Study of Mixed Micelles of Taurocholate and 1,2-Ethylene-di-N-*n*-propylcarbamate

YI-HIAN WU and GIALIH LIN*
Department of Chemistry, National Chung-Hsing University, Taichung 402, Taiwan
Fax: (886)(4)22862547; E-mail: gilin@dragon.nchu.edu.tw

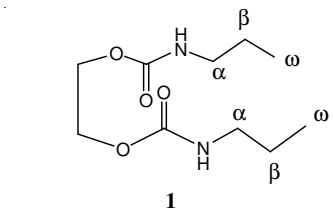
A combined model for the structures of the mixed micelles of taurocholate and 1,2-ethylene-di-N-*n*-propylcarbamate (**1**) has been proposed. Present model suggests that the structures of micelles of taurocholate are equally composed of these four dimeric fragments. Moreover, lipid molecules can only insert into back-to-back dimeric fragments but not into face-to-face dimeric fragments in the mixed micelles of taurocholate and lipid. To test this model, a short-chain analogue of glycerol lipid, 1,2-ethylene-di-N-*n*-propylcarbamate (**1**), was synthesized and was mixed with the micellar taurocholate to form the mixed micelle of taurocholate and compound **1**. From the ^{1}H NMR spectra, the α-methylene protons of compound **1** split after formation of the mixed micelle with taurocholate. Thus, half of compound **1** molecules insert into the anti-parallel back-to-back dimers in the mixed micelle and the other half of compound **1** molecules insert into the parallel back-to-back dimers in the mixed micelle.

Key Words: NMR, Mixed Micelle, Taurocholate, Lipid analogue.

INTRODUCTION

The bile salts are natural occurring, steroidal molecules. The micellization of bile salts play a critical role in the digestion of lipids in animals. Various viewpoints on this topic have been reviewed and become an important issue in surfactant chemistry[1-6]. Synthetic surfactants, fatty acid salts, short-chain phospholipids and other amphiphiles form normal micelle in aqueous solution. The normal micelle consists of the hydrophobic interior and the hydrophilic exterior for favourable interactions with water[1,2]. The critical micelle concentration (CMC) of sodium taurocholate (Fig. 1) has been reported to be 3.4-6.3 mM7. Taurocholate aggregates itself in aqueous solution into a micelle above its CMC.

The structures of micelles of taurocholate have been debated for many years. According to Funasaki's model based on 2D NMR study, anti-parallel back-to-back (ABB) and parallel back-to-back (PBB) dimers (Figs. 2 A&B)[1,5] were proposed as two major fragments in the micellar taurocholate due to strong hydrophobic interactions between two taurocholate molecules[8-11]. However, according to Galantini's model based on X-ray crystal structure, anti-parallel face-to-face (AFF) and parallel face-to-face (PFF) dimers (Figs. 2C & D)[1,5] were proposed as two major fragments



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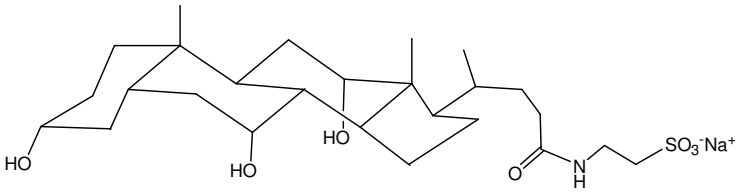
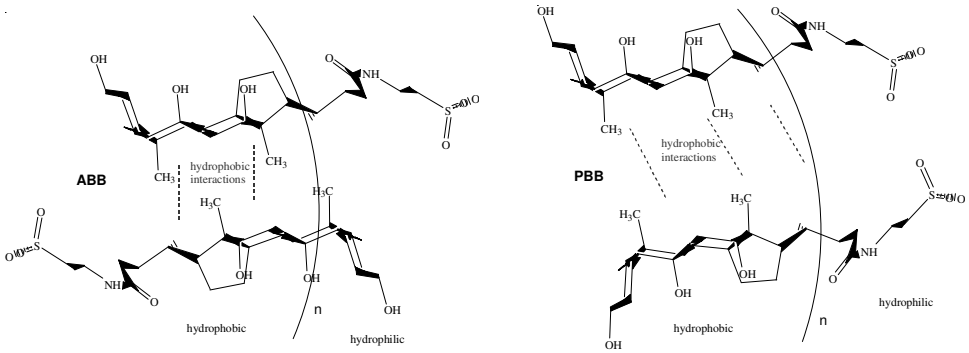


Sodium Taurocholate

Fig. 1. Chemical structures of compound **1** and taurocholate



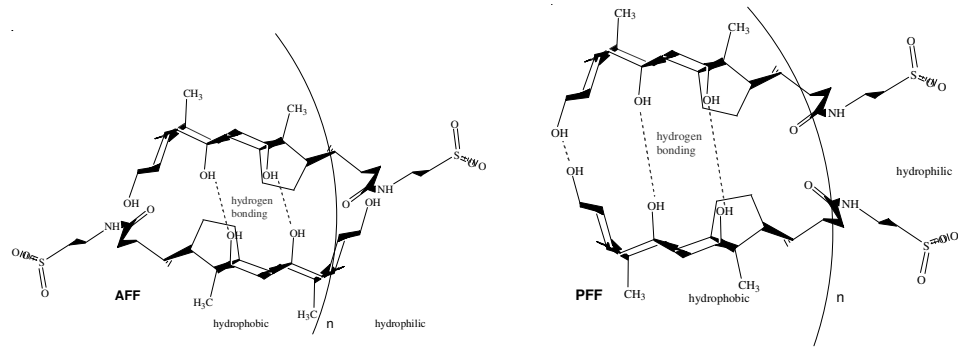


Fig. 2. Four major dimeric fragments in the micelle of taurocholate. (A) anti-parallel back-to-back (ABB), (B) parallel back-to-back (PBB), (C) anti-parallel face-to-face (AFF) and (D) parallel face-to-face (PFF)

in the micelle due to the hydrogen bonding between two taurocholate molecules[12]. Thus, lipid molecules may insert into back-to-back dimers but not into face-to-face dimers. We propose a combined model from these two models for the structures of micelles of taurocholate. Present model suggests that the structures of micelles of taurocholate are equally composed of these four dimeric fragments (Fig. 3). To test this model indirectly, a short-chain analogue of glycerol lipid, 1,2-ethylene-di-N-*n*-propylcarbamate (**1**) (Fig. 1)[13-16], was synthesized and was incorporated into the micellar taurocholate to form the mixed micelle of taurocholate and compound **1**. Thus, we report here the 600 MHz ^{1}H NMR study on the mixed micelles of taurocholate with a short-chain glycerol lipid analog, compound **1**. We find that the α-methylene protons of compound **1** split after formation of the mixed micelle with taurocholate. Therefore, this study suggests that half of compound **1** molecules insert into the ABB dimeric fragments in the taurocholate-1 mixed micelle and the other half of compound **1** molecules insert into the PBB dimeric fragments in the taurocholate-1 mixed micelle.

EXPERIMENTAL

In order to mimic a natural membrane, the mixed micelle in this study consists of sodium taurocholate (TC), the short-chain glycerol lipid analog[13-16] compound **1**, lipase[17-19] substrate *p*-nitrophenyl butyrate (PNPB), bovine serine albumin (BSA) protein and sodium formate (internal reference for NMR) in phosphate buffer (0.1 M, pH 7.0) in 10 % D_2O. Bovine serine albumin, *p*-nitrophenyl butyrate and taurocholate were obtained from Sigma. All other chemicals were of the highest purity available commercially.

Compound **1** (Fig. 1) was synthesized from the condensation of ethylene glycol with two equivalents of the *n*-propyl isocyanate and sodium hydride (NaH) in tetrahydro furan at 25 ºC for 24 h (70-80 % yield)[13-16]. The products were purified by liquid chromatography (silica gel, hexane-ethyl acetate) and characterized by ^{1}H, ^{13}C NMR and high resolution mass spectra.

1,2-Ethylene-di-N-*n*-propylcarbamate (1): ^{1}H NMR ($CDCl_3$, 300 MHz) δ/ppm 0.92 (t, J = 7.2 Hz, 6H, ω-CH_3), 1.52 (sextet, J = 7.2 Hz, 4H, β-CH_2), 3.15 (q, J = 8 Hz, 4H, α-CH_2), 4.25 (s, 4H, OCH_2CH_2O) and 4.80 (s, NH). ^{13}C NMR ($CDCl_3$, 75.4 MHz, assignment from DEPT experiments) δ/ppm 11.1 (ω-CH_3), 23.1 (β-CH_2), 42.7 (α-CH_2), 63.0 (OCH_2CH_2O) and 156.2 (carbamate C=O). Mass spectrum, EI, m/z, exact mass, $[M]^+$ 232.1420 (calculated 232.1424 for $C_{10}H_{20}N_2O_4$).

^{1}H NMR spectra were recorded in D_2O at 600 MHz (Varian ANOVA-600 spectrometer) with an internal reference sodium formate (HCOONa) at 25 ºC. The NMR tubes contained PNPB (0.2 mM) and compound **1** (0.75 mM) with or without taurocholate (7 mM) in sodium phosphate buffer (1 mL, 0.1 M, pH 7.0) containing 10 % D_2O and BSA (0.5 mg) in the presence of an internal reference HCOONa (5 mM) at 25 ºC.

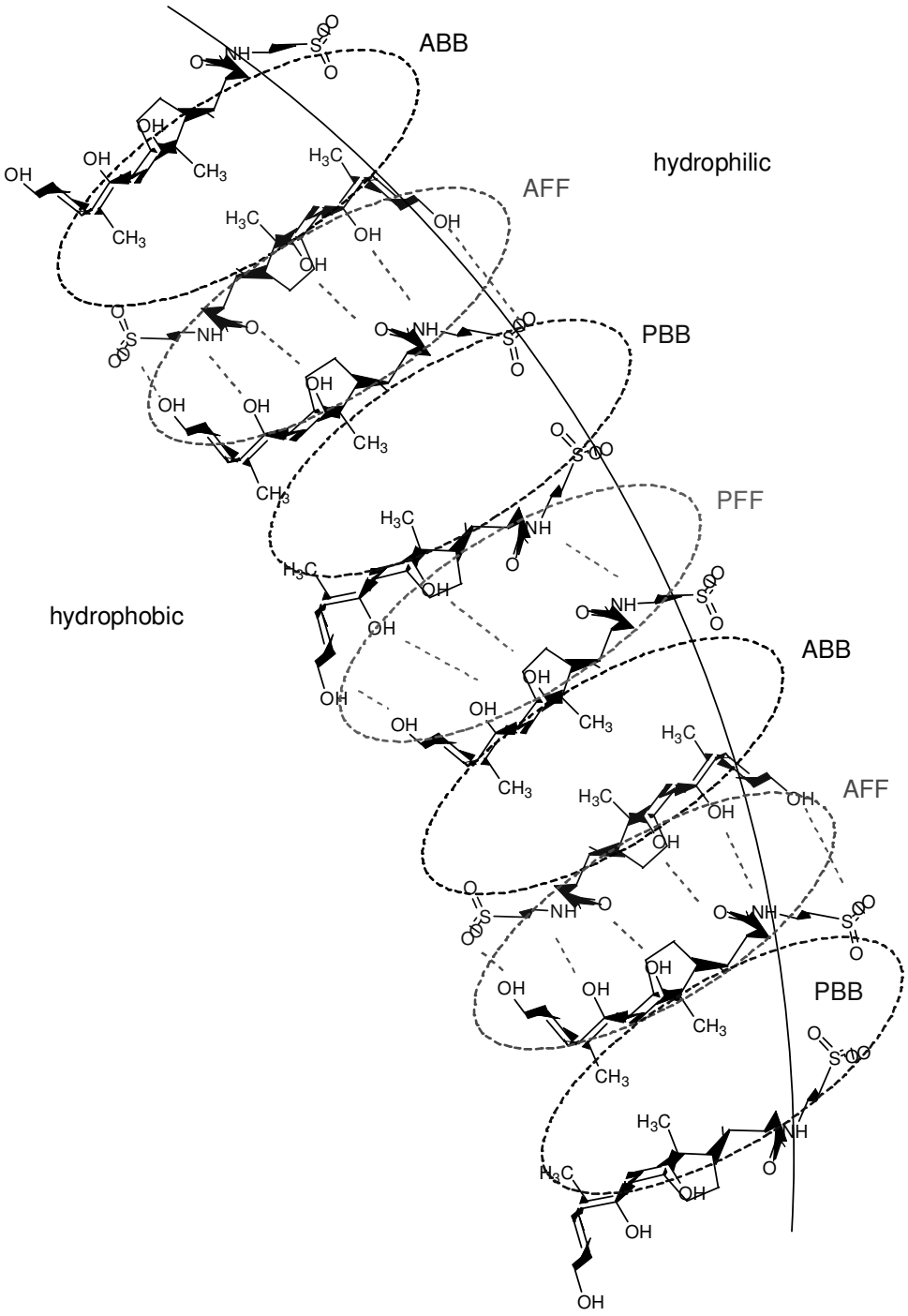


Fig. 3. Proposed structure of micelles of TC. The micelles are equally composed of dimeric fragments of ABB, PBB, AFF and PFF

RESULTS AND DISCUSSION

Combined model for micellar taurocholate: According to Funasaki's model, ABB and PBB dimers (Figs. 2A & B) are proposed as two major fragments in the micellar taurocholate[1,5,8-11]. However, according to Galantini's model based on X-ray crystal structure, AFF and PFF dimers (Figs. 2C & D) are proposed as two major fragments in the micellar taurocholate[1,5,12,20-23]. Structures AFF and PFF are stabilized by hydrogen binding between hydroxyl groups. Structure AFF is also observed in the X-ray crystal structure[20]. However, structures AFF and PFF are exclude from micellar taurocholate from 2D NMR studies[8,9]. There are very few spaces to adopt compound **1** between two taurocholate molecules because of hydrogen binding between hydroxyl groups of two face-to-face taurocholate molecules. Thus, compound **1** can only insert into structures ABB and PBB but not into structures AFF and PFF in the mixed micelle (Fig. 4). At present, we propose a new model from a combination of these two models for the structures of micelles of taurocholate (Fig. 3). According to present new model, a face-to-face dimeric fragment must exist between two back-to-back dimers. Thus, the structures of micellar taurocholate are equally composed of dimeric fragments of ABB, PBB, AFF and PFF. Therefore, present model may stop the debate between Funasaki *et al.* and Galantini *et al.* Moreover, according to present model, lipid molecules may insert into back-to-back dimers but not into face-to-face dimers due to the strong hydrogen bonding in the latter structures (Fig. 4)[12,20-23].

Mixed micelle of compound 1 and taurocholate: The concentration of taurocholate (7 mM) in this study is above its CMC (3.4-6.3 mM)[7]. Therefore, taurocholate molecules aggregate as micelles at the concentration of 7 mM in aqueous solution. On the other hand, low concentration of compound **1** (0.75 mM) may mix homogenously well with micellar taurocholate to form the mixed micelle of taurocholate and compound **1**. Chemical shifts, multiplicities and proton numbers of ω-methyl, α-methylene and β-methylene protons of compound **1** in the absence or presence of micellar taurocholate are summarized in Table-1 from 600 MHz ^{1}H NMR spectra.

TABLE-1
CHEMICAL SHIFTS, MULTIPLICITIES and PROTON NUMBERS OF COMPOUND **1** IN THE ABSENCE OR PRESENCE OF MICELLAR TAUROCHOLATE (TC)[a]

	α-CH_2	β-CH_2	ω-CH_3
1	3.08 (t, 4H)	1.49 (sextet, 4H)	0.89 (t, 6H)
1 in micellar TC	3.08 (t, 2H): ABB[b] 3.13 (t, 2H): PBB	1.49 (sextet, 4H)	0.91 (t, 6H)

[a]All H-C-C-H coupling constants are about 7 Hz, ^3J = 7 Hz.
[b]These moieties are proposed to incorporate into fragments ABB and PBB of the micellar TC (Fig. 6).

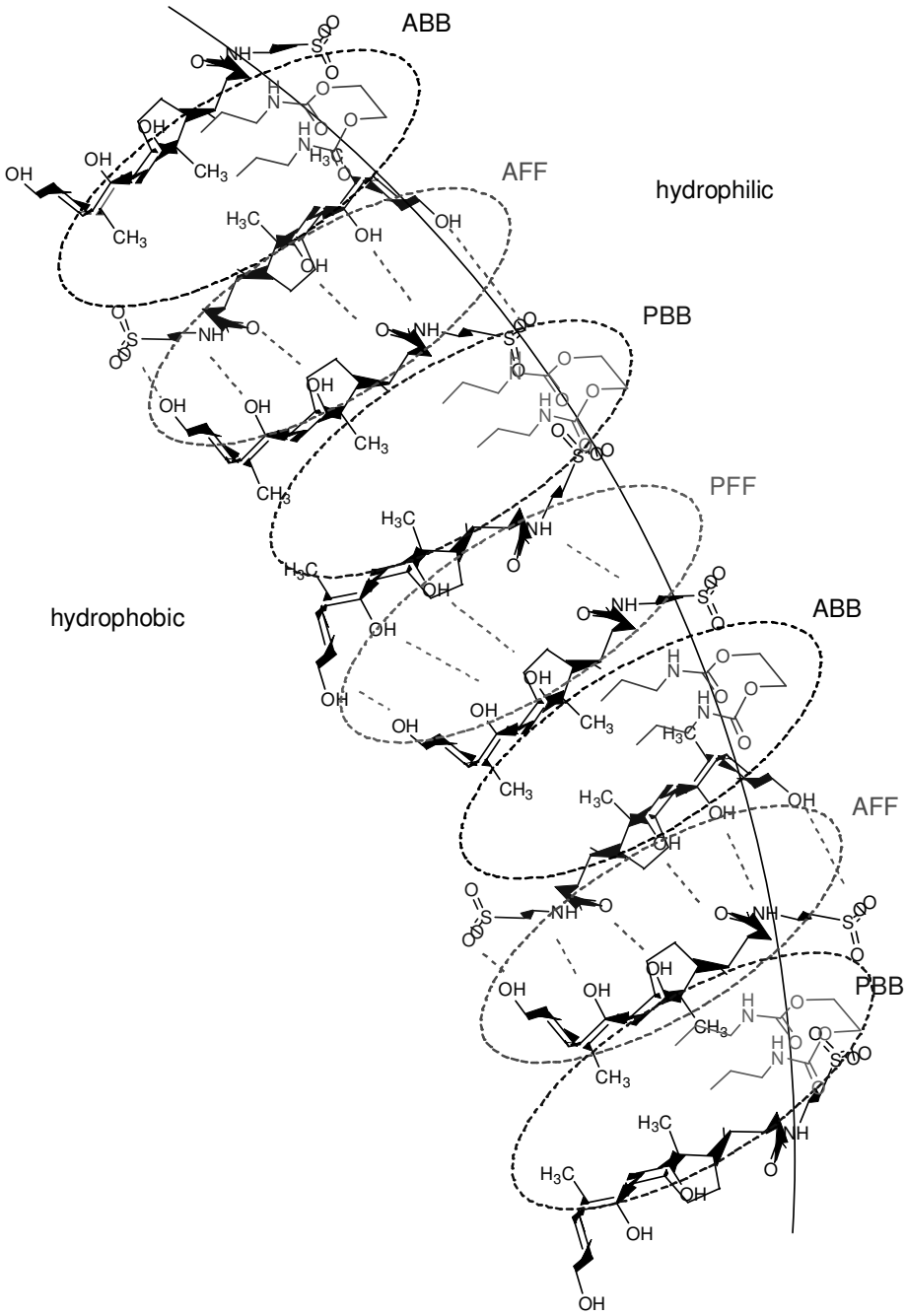


Fig. 4. Proposed structure of the mixed micelles of TC and compound **1**. The mixed micelles are equally composed of dimeric fragments of ABB, PBB, AFF and PFF. Compound **1** molecules incorporate into dimeric fragments of ABB and PBB in the mixed micelle

α-Methylene protons of compound 1 in the mixed micelle: The triplet at 3.08 ppm (J = 7 Hz) which is assigned to be the α-methylene protons of compound **1** splits into two equal-intensity triplets (J = 7 Hz) at 3.08 and 3.13 ppm after formation of the mixed micelles with taurocholate (Fig. 5) (Table-1). Compound **1** molecules insert into ABB and PBB dimeric fragments but not into AFF and PFF dimeric fragments in the taurocholate-compound **1** mixed micelle (Fig. 4). The triple at 3.08 ppm (Fig. 5 bottom) is assigned to be the α-methylene protons of compound **1** in the ABB dimeric fragment of the taurocholate-compound **1** mixed micelle due to the lack of any significant hydrophobic interactions between two steroidal rings of taurocholate molecules in the ABB fragment and the α-methylene group of compound **1** (Fig. 6A). On the other hand, the triple at 3.13 ppm (Fig. 5 bottom) is assigned to be the α-methylene protons of compound **1** in the PBB dimeric fragment of the taurocholate-compound **1** mixed micelle due to strong hydrophobic interactions between two steroidal rings of taurocholate molecules in

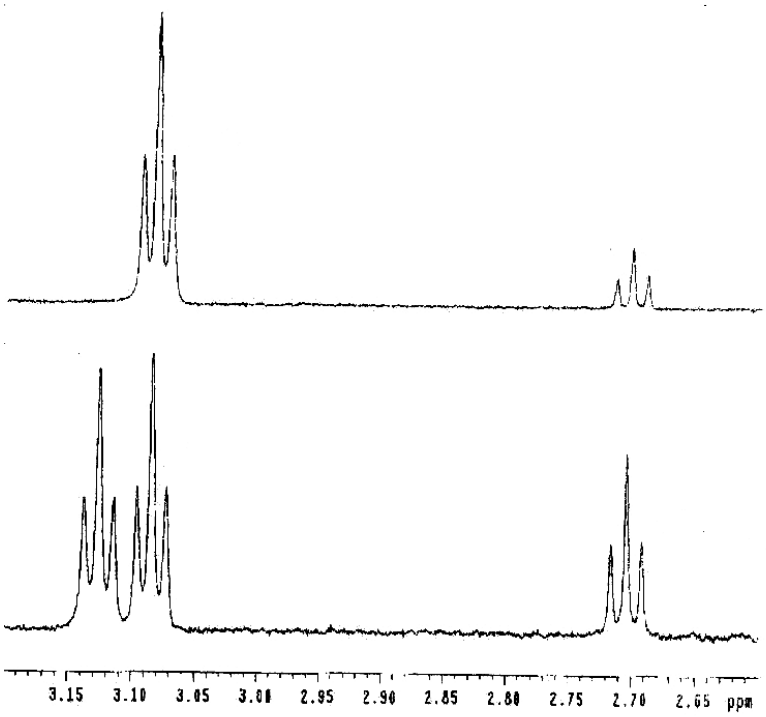


Fig. 5. Partial ^{1}H NMR spectra for α-CH_2 of compound **1** (0.75 mM) containing PNPB (0.2 mM) and BSA (0.5 mg) in sodium phosphate buffer (1 mL, 0.1 M, pH 7.0) in 10 % D_2O in the presence of an internal reference HCOONa (5 mM) in the absence or presence of TC (7 mM) at 25 ºC. In the absence of TC (top), the triplet at 3.08 ppm (J = 7 Hz) was assigned to be the α-CH_2 of compound **1** and the triplet at 2.70 ppm (J = 7 Hz) was assigned to be the α-CH_2 of PNPB. In the presence of TC (bottom), two triplets at 3.08 (J = 7 Hz) and 3.13 ppm (J = 7 Hz) were assigned to be the α-CH_2 of compound **1** in structures ABB and PBB, respectively, of the mixed micelles and the triplet at 2.70 (J = 7 Hz) was assigned to be the α-CH_2 of PNPB

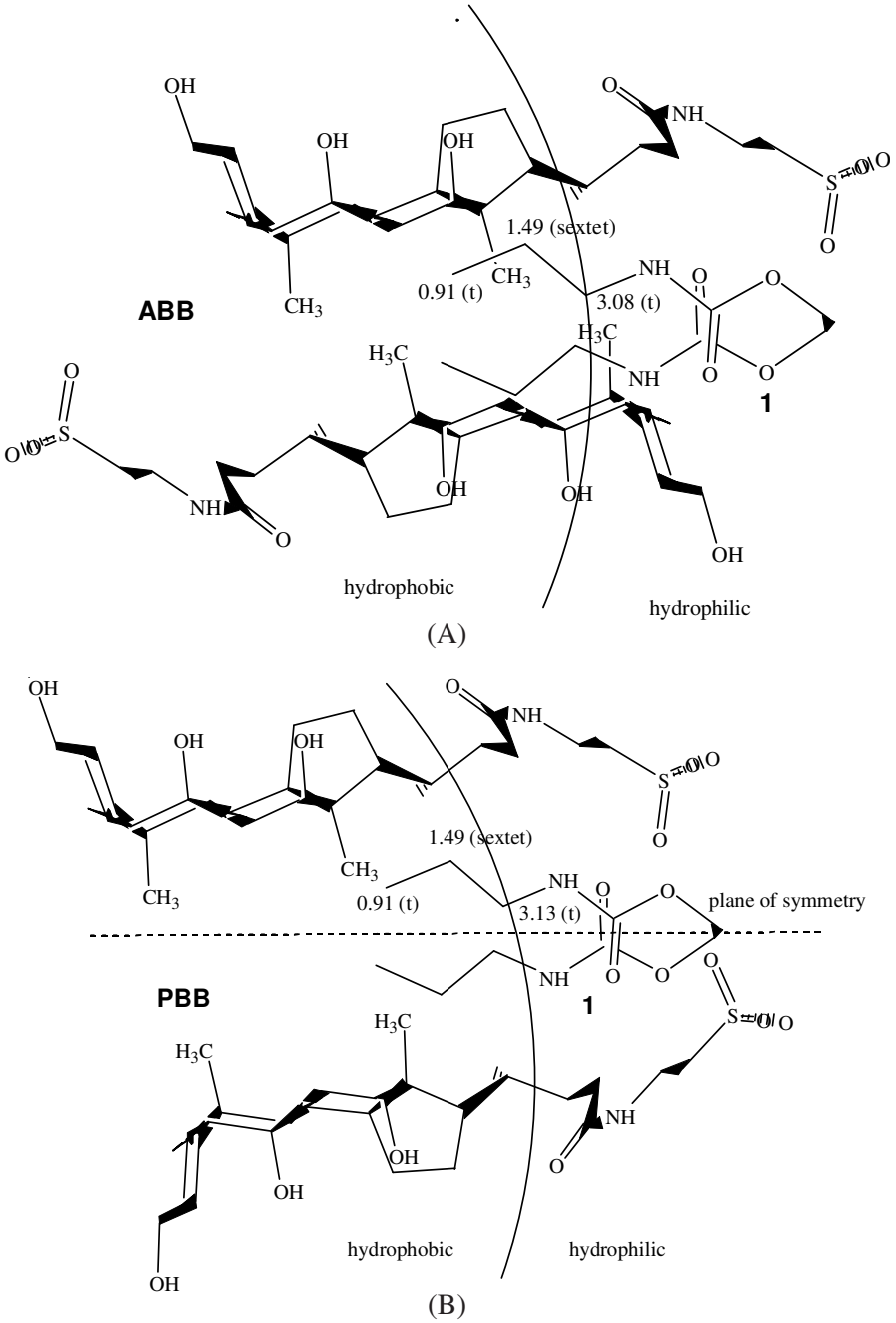


Fig. 6. Proposed interactions between compound **1** and dimeric fragments of (A) ABB and (B) PBB of the micellar TC. (A) Hydrophobic interactions between the steroidal ring of TC and the carbamate moieties of compound **1** are weak. (B) Hydrophobic interactions between the steroidal ring of TC and the carbamate moieties of compound **1** are strong. There is a plane of symmetry in this structure

the ABB fragment and the α-methylene group of compound **1** (Fig. 6B). It is possible that the intermolecular hydrophobic interactions between the α-methylene moiety of compound **1** and the hydrophobic region of taurocholate in structure PBB are stronger than those between the α-methylene moiety of compound **1** and the hydrophobic region of taurocholate in structure ABB (Fig. 6A). In other words, strong hydrophobic interactions may result in the fact that the chemical shift of the α-methylene protons in structure PBB of the mixed micelle (Fig. 6B) shifts more than that in structure ABB of the mixed micelle does after the formation of the mixed micelle (Fig. 6A).

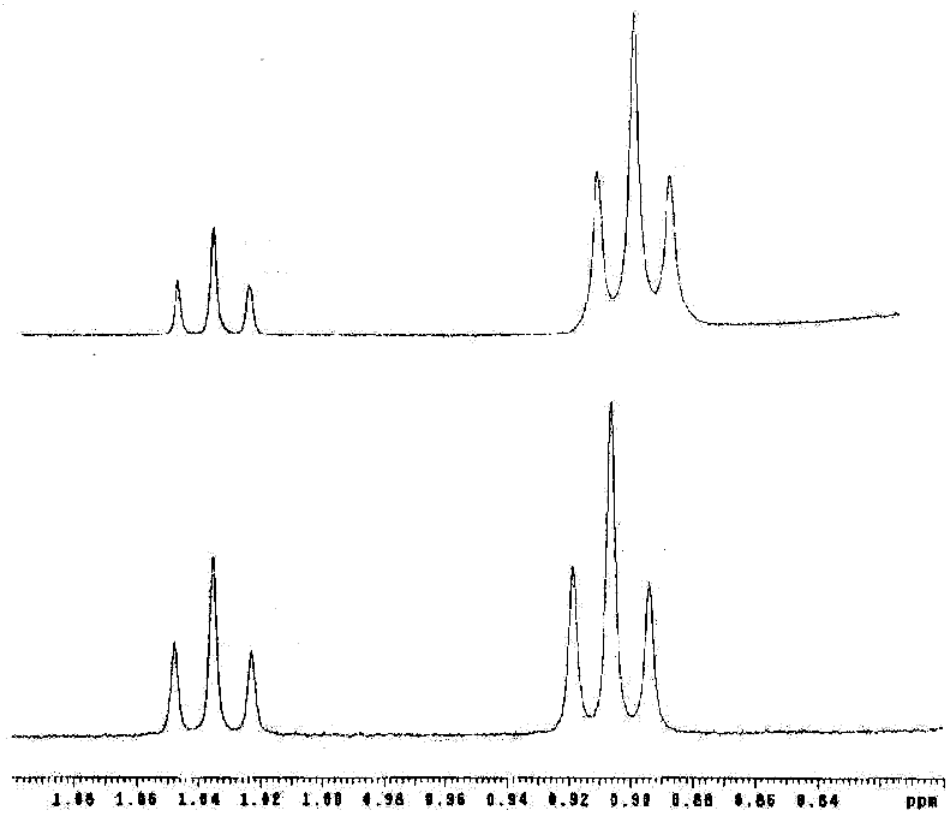


Fig. 7. Partial ^{1}H NMR spectra for the ω-CH_3 protons of compound **1** (0.75 mM) of the solution containing PNPB (0.2 mM) and BSA (0.5 mg) in sodium phosphate buffer (1 mL, 0.1 M, pH 7.0) containing 10 % D_2O in the presence of an internal reference HCOONa (5 mM) in the absence or presence of TC (7 mM) at 25 ºC. In the absence of TC (top), the triplet at 0.89 ppm (J = 7 Hz) was assigned to be the ω-CH_3 of compound **1** and the triplet at 1.03 ppm (J = 7 Hz) was assigned to be the ω-CH_3 of PNPB. In the presence of TC (bottom), the triplet at 0.91 (J = 7 Hz) was assigned to be the ω-CH_3 of compound **1** in the mixed micelles and the triplet at 1.03 (J = 7 Hz) was assigned to be the ω-CH_3 of PNPB

β-Methylene and ω-methyl protons of compound 1 in the mixed micelle: The sextet at 1.49 ppm (J = 7 Hz) is assigned to be the β-methylene protons of compound **1** (Table-1). This sextet does not change its chemical shift and remains the sextet after formation of mixed micelle with micellar taurocholate. The triplet at 0.89 ppm (J = 7 Hz) is assigned to be the ω-methyl protons of compound **1** and the signal retains the triplet (J = 7 Hz) but shift to 0.91 ppm after formation of the mixed micelle with taurocholate (Fig. 7) (Table-1). Therefore, the ω-methyl and β-methylene groups of compound **1** in both structures ABB and PBB in the mixed micelles may rotate very fast around the C-C axis.

Methylene and methyl protons of PNPB in the mixed micelle: *p*-Nitrophenyl butyrate (PNPB) is widely used as a short-chain lipid analog of a lipase substrate[13-16]. PNPB has been reported to incorporate into the mixed micelles of Triton-X 100 or taurocholate[16]. In this study, however, all methylene and ω-methyl protons of PNPB do not change chemical shifts and multiplicities after mixing into the mixed micelle of taurocholate and compound **1** (Figs. 5 and 7). Therefore, hydrophobic or hydrophilic interactions between methylene and ω-methyl groups of PNPB and the taurocholate-compound **1** mixed micelles can not be detected from 600 MHz ^{1}H NMR spectra.

Back-to-face structures in the mixed micelle: It is no doubt that anti-parallel back-to-face (ABF) and parallel back-to-face (PBF) are considered as two minor fragments in micellar taurocholate (Fig. 2)[5,8,9,12,20-23]. Thus, low concentrations of structures ABF and PBF in the mixed micelle of taurocholate and compound **1** will be under the detection limit of 600 MHz 1H NMR spectroscopy.

Two-dimensional structures in the mixed micelle: So far we only discussed about one-dimensional aggregates for the compound **1**-taurocholate mixed micelle (Fig. 4). The shape for one-dimensional aggregates of the mixed micelle looks like a ring in a spherical micelle (three-dimensional structure). Therefore, two-dimensional structures of the mixed micelles will be the side-by-side aggregations of these rings (Fig. 8). Major interactions in the two-dimensional aggregates are hydrophobic interactions between two side-by-side steroidal rings of taurocholate molecules and hydrophilic interactions between polar head groups.

Conclusion

We propose a novel model for structures of micellar taurocholate and may stop the debates between Funasaki *et al.* and Galantini *et al.* Based on our model, the micelles of taurocholate are equally composed of dimeric fragments of ABB, PBB, AFF and PFF. Evidences suggest that a glycerol lipid analogue, compound **1** inserts into dimeric fragments ABB and PBB in the mixed micelle from ^{1}H NMR studies (Table-1). To the best of our knowledge, this paper clearly states a novel structure for the mixed micelle of taurocholate with a glycerol lipid analogue. This study also high lights on the structural elucidation for more complicated bilayers and membranes.

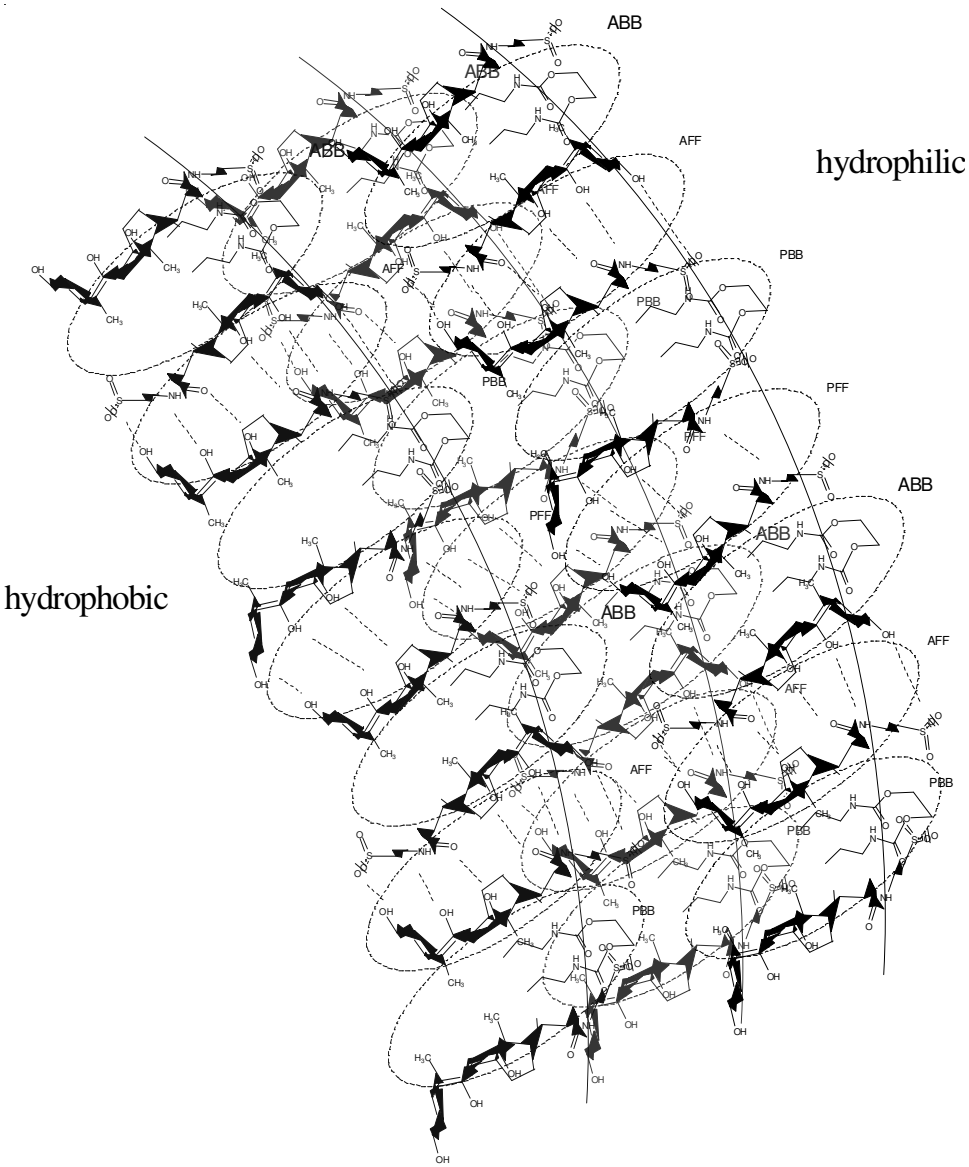


Fig. 8. Proposed two-dimensional structure of the mixed micelles of TC and compound 1. Major interactions between one-dimensional structures are proposed as hydrophobic interactions between two side-by-side steroidal rings of TC molecules and hydrophilic interactions between polar head groups.

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