# Conformational Characterization of α-Mycolic Acid in a Monolayer Film by the Langmuir−Blodgett Technique and Atomic Force Microscopy

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Conformational changes of α-mycolic acid (α-$MA_{MAI}$, isolated from *Mycobacterium avium*−*M. intracellulare* complex) in its monolayer at different surface pressures were investigated by using the Langmuir−Blodgett (LB) technique and atomic force microscopy (AFM). Three representative monolayers that correspond to the gas, liquid-expanded, and solid states were prepared and transferred onto freshly cleaved mica. The monolayer LB films of α-$MA_{MAI}$ were examined by AFM in an angstrom resolution in the thickness direction. The observation of topography and cross section of the monolayers proposed a series of schematic pictures for the lateral arrangement of the alkyl chains of α-$MA_{MAI}$ in the monolayer at different surface pressures. The proposed molecular conformations were consistent with the surface pressure−area isotherm. The results suggested that the α-$MA_{MAI}$ molecules partially took a flat-on stance in the gas-state monolayer and that the full extension of the longer alkyl chain of the molecule in the monolayer was possible only when the monolayer was highly compressed to the solid state.

## Introduction

Mycolic acids or high-molecular-weight 1-alkyl branched 2-hydroxy fatty acids are characteristic components of the mycobacterial cell envelope, and those of slow-growing mycobacteria contain 70−90 carbons. Usually, they contain two functions selected from disubstituted cyclopropyl and olefinic groups or one such group and an oxgen function selected from keto, methoxy, and ester groups in the longer chain of the asymmetric two alkyl chains. These mycolic acids are present in the extractable lipids in the cell surface mostly as trehalose esters and also in the bound form as an acid component sterified to 5-hydroxy groups of the terminal and 2-linked arabinofuranosyl residues of the terminal [β-Araf-(1→2)-α-Araf(1→)]$_2$-3,5-Araf units of the arabinogalactan attached to the shape-forming peptidoglycan of the cell.[1] The electron micrograph of ultrathin sections of mycobacterial cell envelope revealed the presence of an electron transparent layer that consisted of hydrophobic components.[2,3] In this layer, it is suggested that the mycolic acids which amount to, for example, 34 wt % of the cell-wall skeleton in *Mycobactrium microti*[4] are orderly arranged. This orderly packing of the mycolic acids is considered to contribute to the rigidity of the cell-wall structure and also to some important functions such as transportation of hydrophobic antibiotics.

Several authors have proposed schematic models of cell envelope structure to show how the mycolic acids and other lipid components are possibly arranged in the electron transparent layer.[5−7] According to the currently accepted structural model for the mycobacterial cell envelope as reviewed by Draper,[8] the two long hydrocarbon chains of the mycolic acids are arranged orderly in parallel and normal to the cell-wall surface. Nonetheless, the mycolic acids contain two intrachain groups in the major chain, which vary in types and stereochemistry. These groups might have some effects on the compact arrangement of the alkyl chains of the mycolic acid.

Detailed analysis of the surface behavior of the mycolic acid layer is expected to provide an approach to the elucidation of the effects of such intrachain groups on the mycolic acid arrangement and its exact nature in the cell envelope. ESR studies with spin-labeled stearic acid revealed that the presence of a cis double bond or *cis*-cyclopropane ring increased the fluidity of a liquid-crystalline bilayer comprised of long chain fatty acids.[9] A comprehensive study by Durand et al.[10] was carried out on trehalose dimycolate (TDM) and methylglycoside by changing the number of hydrocarbon chains attached to the sugar residues and their chain lengths. They found that TDM formed a stable monolayer on water. The surface property of the monolayer was governed by the long hydrocarbon chains as in dipalmitoylphosphatidylcholine (DPPC) monolayer, and the surface property was not

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significantly affected by the sugar residues. Further, it was suggested that the molecular condensation in a mixed monolayer of TDM and DPPC depended on the number of hydrogen bondings formed between the hydroxyl functions of sugars and phosphate.

TDM monolayers were also investigated by Retzinger et al.,[11] in which the chain folding was discussed by way of the monolayer thickness estimated by the cross-section area and the molecular density of the monolayer. This discussion was made on an assumption that the mycolate residue has a folding part which results in six vertically packed alkyl chains in a molecule. In fact, the thickness of about 2.6−3.0 nm by their estimation was consistent with the folded model.

On the other hand, Almog et al. performed surface pressure−area ($\pi-A$) isotherm measurements for a monolayer of TDM isolated from *M. tuberculosis* H37Rv strain.[12] They analyzed the isotherms via estimation of compressibility that is evaluated by $(-1/A)(dA/d\pi)$ and excess Gibbs free energy. They concluded that the surface properties of TDM monolayers largely depend on the asymmetry of the chain lengths of the mycolate residues, which suggested again that the longer chain folds at a kink point to form the six vertically packed alkyl chains in a TDM molecule.

In the present study, we have found that α-mycolic acid (α-$MA_{MAI}$) from *M. avium−M. intracellulare* (MAI) complex formed stable monolayers on a water surface (Langmuir films or L films) and that they were readily transferred onto a mica plate as Langmuir−Blodgett (LB) monolayer films.[13,14] The topography of the LB films was characterized by atomic force microscopy (AFM),[15] which enables us to discuss the thickness changes of the LB films. The AFM images clearly showed the appearance of some characteristic molecular domains when the surface pressure of the monolayer was increased and revealed the thickness changes of the domains. The quantitative AFM cross-section analysis suggested that the molecular conformations of α-$MA_{MAI}$ changed greatly by changing its chain-folding and that the cross-section area of the proposed models were consistent with the limiting surface area by the $\pi-A$ isotherm.

Our study also demonstrated that α-$MA_{MAI}$ with no sugar residues had different physical properties from those of TDM reported and that the molecular forms with fully extended alkyl chains could be prepared only when the monolayer was compressed to a very high surface pressure.

## Experimental Section

**Preparation and Characterization of α-Mycolic Acid.** α-Mycolic acid methyl ester was prepared from defatted freeze-dried cell mass of MAI complex strain KK45 (obtained from the Culture Collection of Research Institute of Tuberculosis, Kiyose, Tokyo) by alkaline hydrolysis followed by methylation in the usual manner.[16] For purification, silica gel column chromatography and preparative thin-layer chromatography performed on Merck 5744 plates with diethyl ether/hexane (6:100 v/v) were used. The α-mycolic acid methyl ester was hydrolyzed with KOH in 2-propanol as described,[17] and the resulting acid was subjected to silica gel column chromatography with hexanes−ethyl acetate (5:1 v/v) to remove the epimer produced during the hydrolysis and to prepare the α-mycolic acid specimen (α-$MA_{MAI}$).

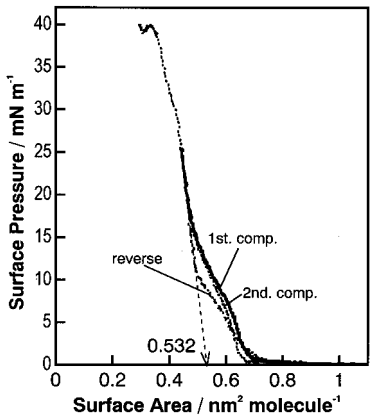
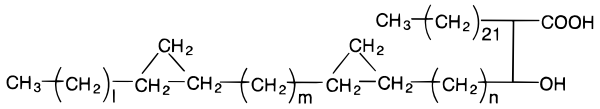



**Figure 1.** Cyclic surface pressure−area ($\pi-A$) isotherms of a monolayer of α-$MA_{MAI}$ on pure water measured at 25 °C.

**Chart 1. Molecular Structure of α-$MA_{MAI}$**




EI/MS of the α-$MA_{MAI}$ methyl ester showed that the 1-alkyl chain of the mycolate was mainly of 23 carbons as reported,[18] and the carbon number, *l*, in Chart 1 was mostly 19. MALDI-TOF/MS showed the molecular weights of the major components of $\alpha_{MAI}$ were 1165 and 1193 (i.e., carbon numbers, *m* plus *n*, in Chart 1 are 29 and 31, respectively). Its $^1$H NMR spectrum showed the proton signals characteristic of a cis-disubstituted cyclopropyl group at −0.34 ppm (1H), of a trans-disubstituted cyclopropyl group at 0.09−0.14 ppm (3H) and at 0.45 ppm (1H) and of a double bond (mostly trans double bond) at 1.9−2.0 ppm (3H) and at 5.4 ppm (2H). By comparison of the areas of these signals, the ratio between the cis-disubstituted cyclopropyl group, trans-disubstituted cyclopropyl group, and trans double bond in the α-$MA_{MAI}$ was estimated to be 100:14:8, which showed that the dominant components of the present $\alpha_{MAI}$ were $C_{80}$ and $C_{82}$ acids with two cis-disubstituted cyclopropyl groups.

**Monolayer Formation and LB Transfer.** A chloroform solution of α-$MA_{MAI}$ at a concentration of 1.0 mg mL$^{-1}$ was prepared and stored in a glass tube with a Mininert valve. The chloroform solution of 70 $\mu$L was spread with a microsyringe on a pure water subphase in a trough (initially 65 × 14 cm). The pure water was obtained by a Millipore (Bedford, MA) "Milli-Q Labo" water purifier equipped with a final membrane filter with micropores of 0.22 $\mu$m. The electric resistance of the pure water was above 18.3 MΩ cm. After we waited for 15 min to fully evaporate the solvent, the monolayer film was compressed parallel to the water surface. The surface pressure was measured by a Wilhelmy balance with a glass plate. The monolayer experiments were performed on a Kyowa Interface Science (Saitama, Japan) HBM LB film apparatus. The compression speed was 14.0 cm$^2$ min$^{-1}$ (3.91 Å$^2$ min$^{-1}$ molecule$^{-1}$).

The monolayer on the water surface was transferred on a freshly cleaved mica surface (hydrophilic) for the AFM measurements by the LB (vertical dipping) technique.[13,14] The mica size was 1 × 4.5 cm, and the stroke of the LB deposition was 3.5 cm. The lifting speed of the mica plate during the LB deposition was 0.5 cm min$^{-1}$. The transfer ratio was almost unity for every transfer. The LB films were dried with $P_2O_5$ overnight prior to the AFM measurements.

**AFM Measurements.** The AFM images of the LB films were measured on a Digital Instruments (Santa Barbara, CA) Nanoscope III, which was isolated on a hung stone bench to keep the apparatus free from any vibrations. The tapping mode was used for the AFM scanning. The cantilever was made of a crystalline

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silicon tip, and its tapping frequency was 280−410 Hz. The height of steps or holes in the monolayer was evaluated by section analysis.

## Results

The $\pi-A$ isotherms of the α-$MA_{MAI}$ monolayer on water after a cyclic measurement are presented in Figure 1. On the first compression, the isotherm leaves off the surface-area axis at around 1.0 $nm^2$ $molecule^{-1}$. The gas-state monolayer[14] continues to remain until the surface area is reduced to ca. 0.7 $nm^2$ $molecule^{-1}$. During the gas state, the surface pressure was below 0.5 mN $m^{-1}$. On further compression, the surface pressure markedly increases, and a transition region of $(d\pi/dA)_T$, which is an expanded region of the isotherm, appears at around 0.6 $nm^2$ $molecule^{-1}$. Above 17.0 mN $m^{-1}$, the surface pressure goes up linearly until the pressure attains 25.0 mN $m^{-1}$.

After the surface pressure attained 25.0 mN $m^{-1}$, the monolayer was expanded by moving the barrier in the opposite direction. The reverse isotherm for the expansion is shown by the dot-broken line in the same figure. The surface pressure goes down linearly to ca. 10.0 mN $m^{-1}$. On further expansion, a similar transition region appears, and the surface pressure goes down to 0.5 mN $m^{-1}$ or lower at around 0.7 $nm^2$ $molecule^{-1}$. In this manner, the monolayer showed a small hysteresis in the cyclic measurement.

The monolayer was compressed again after the monolayer was expanded to 1.2 $nm^2$ $molecule^{-1}$. The isotherm on the second compression is presented by the dashed curve in Figure 1. It is almost identical to that of the first compression, except a slight difference in the lower surface-pressure region. Of note is that the second-compression isotherm in the solid state (linear part) is identical to the first-compression one. The results strongly suggest that the monolayer is not collapsed at 25.0 mN $m^{-1}$ on the first compression, although the cyclic isotherm presents the hysteresis. The hysteresis was reproduced in the replicated cyclic measurements.

To characterize the monolayers, three representative monolayers that correspond to the gas, liquid-expanded, and solid states were prepared at 0.5, 5.0, and 18.0 mN $m^{-1}$, respectively, and transferred onto mica plates by the LB technique. The surface topography and thickness of the LB films on the micas were investigated by AFM.

Figure 2a−c shows the AFM images for the LB monolayers on mica. Figure 2a reveals that the monolayer of α-$MA_{MAI}$ prepared at 0.5 mN $m^{-1}$ has a number of holes here and there, and some of them expose bare mica surface. The cross-section image obtained on the straight line in the AFM image (the right panel in Figure 2a) demonstrated that the average thickness of the monolayer was about 2.6−2.8 nm. The analytical results are summarized in Figure 3.

The AFM image of the α-$MA_{MAI}$ monolayer LB film prepared at 5.0 mN $m^{-1}$ (Figure 2b) presents a largely different result. The image shows a terracelike topography, which indicates that the monolayer consists of at least two domains. In this image, deep holes are few. The section analysis in the right panel suggests that there are at least three steps on the mica surface (Figure 3). The more significant difference in thickness levels was estimated to be ca. 1.4−1.7 nm, and the other smaller difference in thickness was estimated to be about 0.6 nm.

The third AFM image of the same monolayer on mica prepared at 18.0 mN $m^{-1}$ in Figure 2c is definitely different from the former images. Small steps found in Figure 2b are scarcely seen at 18.0 mN $m^{-1}$, and a new terrace topography appears. The size of each domain (terrace) is fairly large. The surface of each terrace was highly smooth as shown by the section analysis, and the surface roughness was estimated to be less than 0.1 nm. The height difference between the steps was found to be ca. 1.1 ∼ 1.2 nm (Figure 3).

## Discussion

Isotherms of various TDMs have been reported, but our results show that mycolic acid itself with no sugar residues can form a stable monolayer. The cyclic measurement of the isotherm for the α-$MA_{MAI}$ monolayer in Figure 1 reveals that α-$MA_{MAI}$ forms a very stable monolayer despite its unique unbalanced structure with a small hydrophilic group and a large asymmetric hydrophobic moiety. Repeated cyclic measurements revealed that the monolayer was not collapsed at surface pressures below 35.0 mN $m^{-1}$.

The limiting molecular area of the isotherms obtained by extrapolation of the linear part is 0.53 $nm^2$ $molecule^{-1}$, which is quite similar to the limiting molecular area of DPPC monolayer (ca. 0.52 $nm^2$ $molecule^{-1}$ at 25 °C). In a highly compressed monolayer, DPPC is known to take a highly organized molecular arrangement with an all-trans zigzag conformation. Since both α-$MA_{MAI}$ and DPPC contain two hydrocarbon chains in each molecule, the present result analogously suggests that α-$MA_{MAI}$ also takes a highly organized molecular conformation in the solid-state monolayer, although α-$MA_{MAI}$ has two largely asymmetric hydrocarbon chains.

The hysteresis observed during the cyclic isotherm measurements suggests that the transition process from one molecular conformation to another by the monolayer compression is not simple: the hydrophobic property of the longer chain in α-$MA_{MAI}$ may play some role in it. Nonetheless, the good reproducibility found in the replicated cyclic measurements suggests that molecular rearrangement upon the monolayer compression is considerably reversible. Therefore, it can be speculated that α-$MA_{MAI}$ in the highly compressed monolayer does not change its molecular shape significantly even in a less compressed monolayer, when the surface pressure is above 10.0 mN $m^{-1}$. On further expansion, however, the extended molecules may cause chain folding in the longer chain, due to the adequate space produced in the monolayer after the expansion. To verify the speculation, the AFM results will be referenced.

The many holes found in the AFM image of the α-$MA_{MAI}$ monolayer prepared at 0.5 mN $m^{-1}$ (Figure 2a) indicate that the monolayer molecules are not packed fully. This means that the monolayer does not fully cover the substrate surface and that bare mica surface is directly touched by the AFM tip. This property enables us to measure the absolute height of the monolayer (thickness) from the mica surface. The section analysis estimates the thickness of the monolayer to be about 2.6−2.8 nm. Nevertheless, since it is possible that there are flat-on molecular species (Figure 4a) on the mica at a very low surface pressure, the bare surface may be partly covered with the flat-on molecules. In this case, the thickness of the monolayer by the section analysis may be less than the true thickness by about 0.2−0.3 nm (Figure 3).

The analysis also suggests that the monolayer surface is coarse with roughness of ca. 0.5−0.7 nm. This may be, however, due to experimental error, since the AFM tip does not reach the mica surface when the diameter of a hole is smaller than the diameter of the AFM tip. Therefore, this surface roughness is not discussed in the present study.

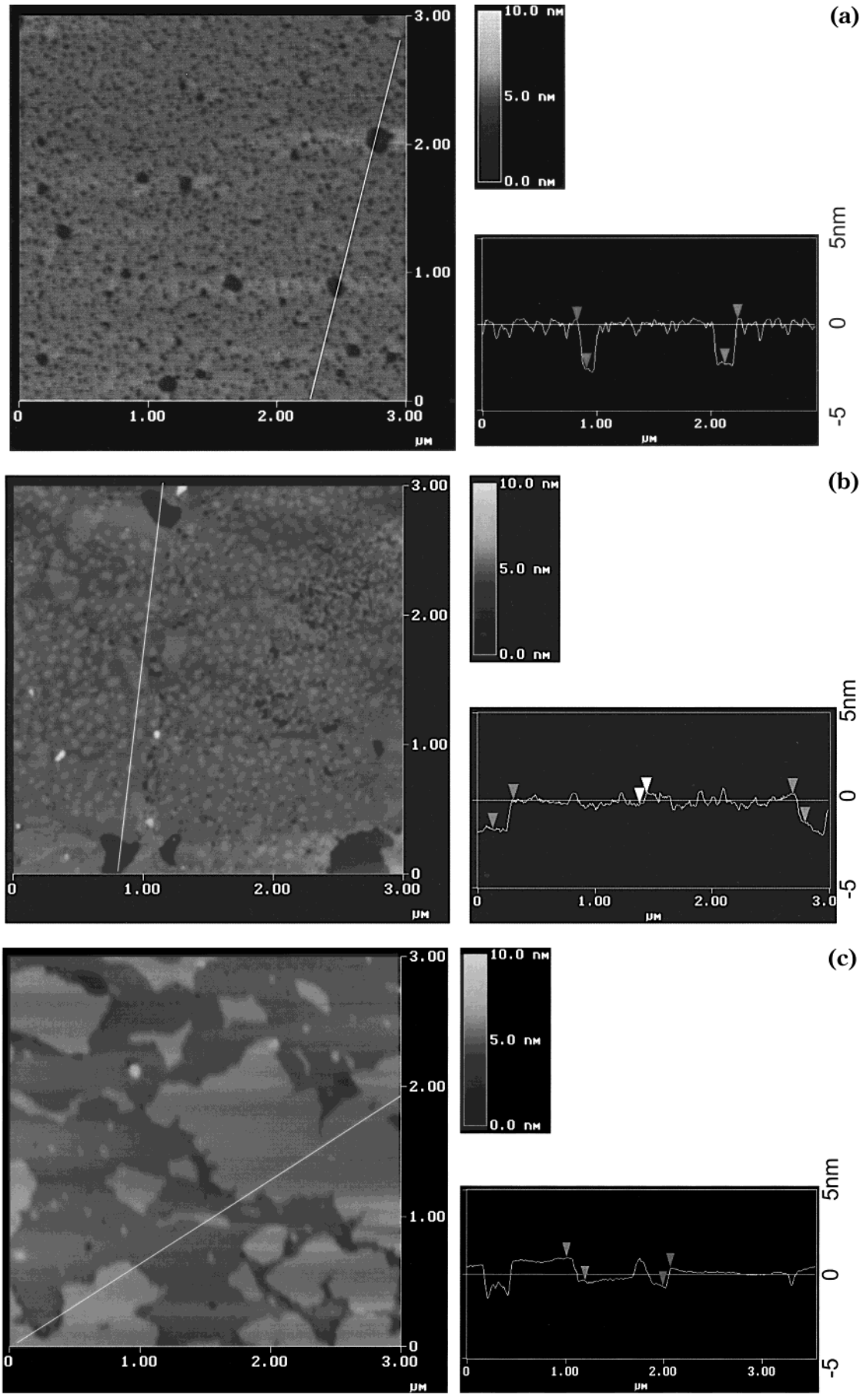


**Figure 2.** AFM images of α-$MA_{MAI}$ monolayer on mica prepared at (a) 0.5, (b) 5.0, and (c) 18.0 mN $m^{-1}$. Each image accompanies a cross section on the line.

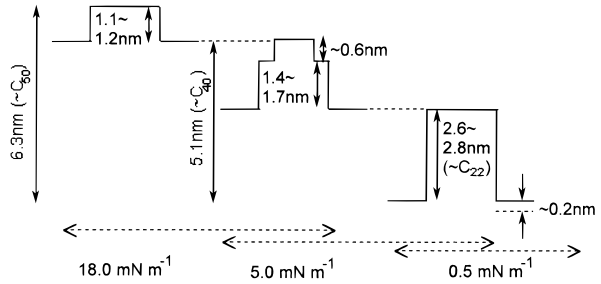


**Figure 3.** Schematic cross-section profiles by AFM.

According to Abrahamsson and von Sydow,[19] the typical distance between alternate carbon atoms in an alkyl chain of zigzag form is 0.254 nm, from which the distance between two adjacent carbons in the direction of the axis of the alkyl chain is calculated to be 0.127 nm. Accordingly, the length of the shorter chain of the mostly $C_{23}$ alkyl chain of α-$MA_{MAI}$ with all-trans zigzag conformation is estimated to be 2.79 nm or when the COOH residue (0.53 nm)[20] is included, to be 3.32 nm. The thickness of the monolayer in the gas state, 2.6−2.8 nm (or more if flat-on molecules are taken into account), estimated by AFM seems to be reasonable when the molecule in Figure 4b tilts at about 25−32° from the normal to the surface. It should be noted here that the shorter alkyl chain in this form is not folded.

The mnemonic molecular conformations suggested by the discussion above are presented by Figure 4a,b. Figure 4a gives an image of the flat-on molecule whose alkyl chains lie flat on the mica surface. This species is expected to possess a very large area and a less significant thickness. Figure 4b shows a partly upright molecular form, in which the longer alkyl chain is folded at least once, with its larger portion mostly in a disordered state. Nonetheless, the lower part of the longer chain is ordered and parallel to the shorter alkyl chain, since this arrangement provides a more stable form that owes to the hydrophobic interaction with the shorter alkyl chain. Of note is that the cross section area of this molecule in this model should correspond to three times the cross section of a hydrocarbon chain or larger. The cross-section of a typical saturated hydrocarbon chain with all-trans zigzag conformation is about 0.2 $nm^2$ $molecule^{-1}$.[21] Therefore, roughly speaking, the molecule in the conformation should have a cross section of 0.6 $nm^2$ $molecule^{-1}$ or larger in the gas state. As expected, in the gas state below 0.5 mN $m^{-1}$, the surface area is larger than 0.66 $nm^2$ $molecule^{-1}$ (Figure 1). This proves that the molecular models (a) and (b) are experimentally acceptable ones.

Figure 2b presents a terrace profile of the α-$MA_{MAI}$ monolayer prepared at 5.0 mN $m^{-1}$. The monolayer molecules are considered to be highly packed, since deep holes are few in this AFM image. To measure the absolute thickness of the monolayer, the bare mica surface was tried to be revealed by scraping the monolayer off with the AFM tip. The scraping was, however, not readily achieved in the Figure 2b film because of the large thickness of the monolayer. This suggests a fact that the molecules in the monolayer at 5.0 mN $m^{-1}$ is much thicker than that at 0.5 mN $m^{-1}$.

The AFM image at 5.0 mN $m^{-1}$ consists of three major domains. One of the domains corresponds to large holes of 1.4−1.7 nm below the continuous domain. On closer inspection, the continuous domain has many terraces of height ca. 0.6 nm (Figure 3).

Schematic pictures illustrated in Figures 4c,d are the possible molecular models in the liquid-expanded state monolayer, which correspond to the AFM results in Figure 2b. In these models, the second cyclopropyl group situated farther away from the carboxyl group forms a turning point to make the longer alkyl chain folded. In Figure 4c, the cyclopropyl group is located at the turning point, while the second carbon in the cyclopropyl group is the folding point in Figure 4d. In both cases, the terminal end of the folded longer chain is in the vicinity of the terminal end of the shorter chain, so that most parts of the chains take a parallel arrangement with the aid of hydrophobic interaction. These molecular models result in a geometrically stable molecular structure as a whole. The terminals of the longer and shorter chains may overlap with each other to some extent, which may yield an

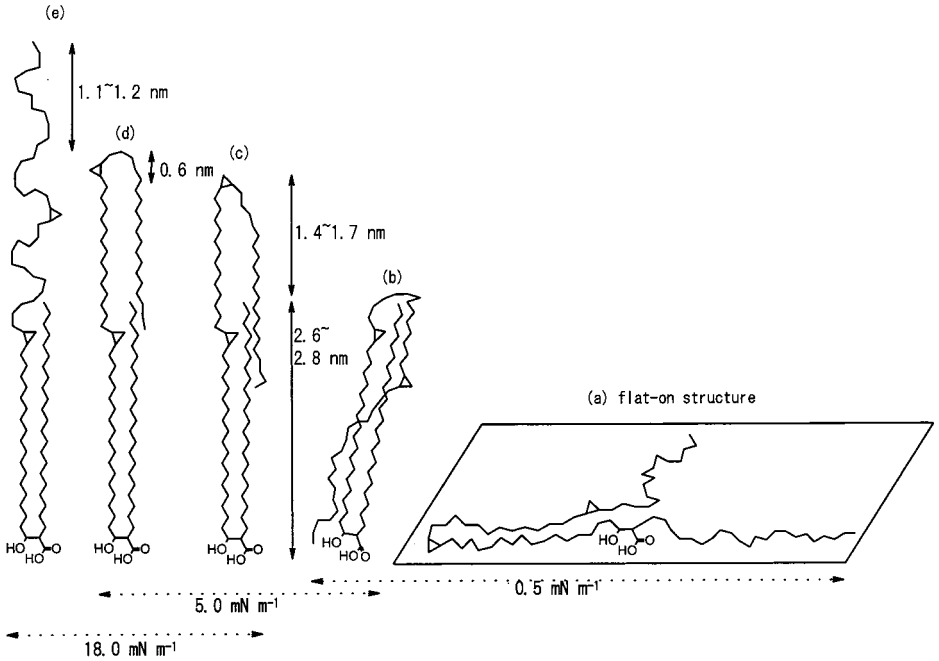


**Figure 4.** Schematic drawings of the α-$MA_{MAI}$ molecule in its monolayer at different surface pressures (a) 0.5, (b) 5.0, and (c) 18.0 mN $m^{-1}$.

increase of the cross section of the molecule. Since the portion of the holes is small, the average cross-section area is expected to be close to 0.6 $nm^2$ $molecule^{-1}$, which corresponds to three times the cross-section area of the hydrocarbon chains. Figure 1 shows that the cross-section area is 0.63 $nm^2$ $molecule^{-1}$ at 5.0 mN $m^{-1}$. Thus, the proposed molecular conformations are considered possible. On the basis of on these models, the total chain length of the molecules estimated from the AFM results (Figure 3) is 4.5−5.1 nm (Figure 3), which agrees with the sizes of both models.

Upon further compression of the monolayer, the turning point (kink) of the longer alkyl chain where the chain folding takes place moves further away toward the tail. This shift of the kink is theoretically verified by molecular mechanics calculation.[22] Therefore, it is possible that the overlap of the shorter and longer chain tails is resolved first, and then a new stable conformation is formed (Figure 4e). In the new conformation, the longer chain has no folding. Of particular note is that a half or a larger part of the extended longer chain is not supported by the parallel-going shorter alkyl chain. In other words, the portion of the longer alkyl chain above the top end of the shorter alkyl chain may be loosely packed in the double-chain space. Therefore, the longer chain is possibly gauche-rich above the first cyclopropyl group. Along this story, the schematic Figure 4e is drawn, in which the total chain length of the molecule is longer than that in Figure 4d by 1.1−1.2 nm (refer to Figure 3).

In this manner, the α-$MA_{MAI}$ monolayer prepared at 18.0 mN $m^{-1}$ was investigated, and it was found to consist of three domains of conformations as shown in Figure 4c−e. In this mixture, the average cross-section area of the molecules should be between the cross-section areas of double and triple chains (0.4−0.6 $nm^2$ $molecule^{-1}$). In the $\pi-A$ isotherm in Figure 1, the surface area at 18.0 mN $m^{-1}$ is found to be 0.48 $nm^2$ $molecule^{-1}$, as expected, which proves that the molecular conformation deduced from the AFM results is a quite reasonable one. The reason the surface area is less than the limiting molecular area (5.3 $nm^2$ $molecule^{-1}$) is that the slope of the solid state in Figure 1 is not steep, which means that the solid-state region is a progressive transition region from a triple-chain molecule to double-chain molecule.

In summary, the fully extended α-$MA_{MAI}$ molecule is possible only when the molecule is laterally compressed by high surface pressure, which makes the monolayer take the solid state.

## Conclusion

Studies of various TDM monolayers have been reported thus far.[10−12] Our present analytical results on the stable monolayers of mycolic acid itself with no sugar residues, however, enabled us to conduct more detailed analysis of the surface character of mycobacterial mycolic acids. The conformation of the α-$MA_{MAI}$ molecule in its monolayer was revealed to change greatly when the surface pressure changed. It was suggested that the position and possibly stereochemistry of the cyclopropyl groups in the longer chain and the hydrophobic interaction between the parallel-going short and long chains are the crucial factors for the possible conformations. The thickness changes of the monolayer were directly depicted by AFM, which is verified by the changes of the molecular cross-section area by the $\pi-A$ isotherm measurements.

The present study shows that the proposed molecular conformation of α-$MA_{MAI}$ with fully expanded longer chain (i.e., no folding as proposed in previous schematic cell envelope models) is possible only when the surface pressure is high, in which the monolayer is in the solid state. This means that the longer chain is unstable in free energy when it is fully extended. The hysteresis found in the cyclic $\pi-A$ measurements may be due to the difference in the changing rate of the molecular conformation toward the more stable free-energy state upon compression and expansion. This difference is understandable, since the molecular conformation is dependent on a force balance between molecular hydrophobic interaction and repulsive forces, the latter of which is caused by the springlike force generated by the longer alkyl chain, since the extended longer alkyl chain is likely to automatically fold at a low surface pressure. This leaf-spring-like property may be a reason to realize the highly condensed packing of mycolic acids in a cell wall of mycobacteria when the mycolic acids with high surface pressure are linked to the polymer sugar network of the arabinogalactan layer.

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