Conformational folding of mycobacterial methoxy- and ketomycolic acids facilitated by \( \alpha \)-methyl trans-cyclopropane groups rather than cis-cyclopropane units

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The oxygenated long-chain mycolic acids from many mycobacteria are characterized by the presence of mid-chain cyclopropane groups, which can have either cis-configuration or trans-configuration with an adjacent methyl branch. To determine the effect of these functional groups on mycolic acid conformation, surface pressure (\( \pi \)) versus mean molecular area isotherms of methoxy- (MeO-) mycolic acids (MAs) from Mycobacterium kansasii, Mycobacterium tuberculosis (Mtb) Canetti and Mtb H37Ra, and of keto-MAs from Mycobacterium avium–intracellulare complex (MAC) and Mtb H37Ra were recorded and analysed. The MeO- and keto-MAs from Mtb H37Ra, containing scarcely any trans-cyclopropyl groups, apparently took no fully folded ‘W-form’ conformations. Keto-MA from MAC, whose trans-cyclopropyl group content is nearly 90 \%, showed a very solid W-form conformation. MeO-MAs from M. kansasii and Mtb Canetti gave stable W-form conformations at lower temperatures and surface pressures and extended conformations at higher temperatures and surface pressures; their W-form conformation was not as stable as expected from their cis-cyclopropyl group content, probably because they had a wide range of constituent homologues. Energy level calculations of cis- or \( \alpha \)-methyl trans-cyclopropane-containing model molecules and computer simulation studies confirmed the superior folding properties of the latter functional unit. The present results were compared with those of MeO- and keto-MAs from Mtb and from Mycobacterium bovis Bacillus Calmette–Guerin (BCG) reported previously. Among the oxygenated MAs, those having higher trans-cyclopropane content tended to take W-form conformations more firmly, implying that the meromycolate proximal intra-chain \( \alpha \)-methyl trans-cyclopropane groups facilitated MA folding more than cis-cyclopropane groups.

INTRODUCTION

Mycobacterial mycolic acids (MAs) are characteristic components of the mycobacterial cell envelope, where major proportions of the acids are covalently bonded to the cell wall penta-arabinosyl units (Minnikin, 1982; Draper, 1998; Dmitriev et al., 2000). As shown in the general structure below, MAs are long chain 2-alkyl branched, 3-hydroxy fatty acids, with two intra-chain groups X and Y in the major chain, termed the meromycolate chain:

\[
\text{CH}_3\text{-}(\text{CH}_2)_p\text{-CHCOOH-CHOH-}(\text{CH}_2)_n\text{-Y-}(\text{CH}_2)_m\text{-X-}(\text{CH}_2)_r\text{-CH}_3
\]

According to the distal intra-chain functional group X, the MAs in this study are classified into three major groups: a non-oxygenated \( \alpha \)-, and two oxygenated groups, methoxy- (MeO-) and keto-MAs. According to the proximal intra-chain group

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### Table 1. Features of MA samples assayed

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* MW: Molecular Weight

† Comparison of cyclopropane: cis : trans is shown.
**Table 1. cont.**

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*Estimation of molecular weight, MW, on the basis of FAB MS data of their meromycolic acids. Numbers underlined refer to the three major components of the sample and those in bold face refer to the peaks needed to constitute 70% of the sample. MWs given are of parent MAs derived from the meromycolic acid MW. Figures in parentheses refer to the segment lengths n/m/l.

†Ratios estimated on the basis of H-NMR spectra.

‡Mtb H37Ra MA consists of two series of MAs. A-series MAs contain one cyclopropane ring, whereas B-series ones have two cyclopropane rings in the meromycolate chain. MW of B-series is 12 higher than that given at the head of each column.
Y, each is further grouped into three main types, with the major type-1 having Y as a cis- or trans-cyclopropane group (Watanabe et al., 2001, 2002). In the present study, type-1 MeO-MA and type-1 keto-MA (hereafter referred to simply as MeO-MA and keto-MA, respectively) were used. It is expected that the structures of these very long mycolic acids, with meromycolate chain lengths of over 50 carbons, will have profound effects on the biological or biophysical cell wall functions. These effects should vary depending on the three dimensional forms or conformations of those MAs forming the outer layer of the mycobacterial cell envelope. This study aims to explore the importance of having either a cis-cyclopropane or an α-methyl trans-cyclopropane as the proximal meromycolate intra-chain group Y.

As reported previously, Monte Carlo calculations showed that most of the MAs from the slow-growing mycobacteria tend to form folded conformations, an energetically more stable form, in which the four methylene chain segments are packed together more or less in parallel, hereafter referred to as a ‘W-form’ conformation (Villeneuve et al., 2005, 2007, 2010). However, the thermodynamic studies of the Langmuir monolayers show that different MAs have different conformational behaviours (Villeneuve et al., 2005, 2007, 2010). Thus, non-oxygenated α-MAs take W-form conformations only in a limited area where both temperatures and surface pressures are very low (Villeneuve et al., 2010). Oxygenated MAs, especially keto-MAs, generally form more stable W-form conformations partly owing to the likely hydration of the keto group on the water surface (Villeneuve et al., 2005, 2007). Our previous monolayer studies showed that the MeO-MAs and keto-MAs from Mycobacterium bovis Bacillus Calmette–Guérin (BCG) and Mycobacterium tuberculosis (Mtb) Aoyama B had distinct conformational behaviours, including different features of the surface pressure (π) vs mean molecular area (A) isotherms (Villeneuve et al., 2005, 2007). The constituent molecules of MeO-MAs and keto-MAs from BCG and those from Mtb are essentially the same, differing only in the ratios of the cis- and trans-cyclopropyl groups. The same applies to the keto-MAs from the two, suggesting a positive effect of the cis- or trans-cyclopropane configuration in the intra-chain group Y on the conformational behaviours (Villeneuve et al., 2005, 2007).

In the present study, to analyse the effect of the cis-cyclopropyl and α-methyl trans-cyclopropyl intra-chain groups on the conformational behaviours, π vs A isotherms were measured at different temperatures for representative samples. The MAs selected were MeO-MAs from Mtb H37Ra, Mtb Canetti and Mycobacterium kansasii and keto-MAs from Mtb H37Ra and Mycobacterium avium–intracellulare complex (MAC), each having different α-methyl trans-cyclopropane contents. To analyse and compare their conformational behaviours, monolayer phase diagrams were prepared by plotting the π vs values, the surface pressure where the conformational transition from W-form to extended conformations took place, and π values, the surface pressure where the phase transitions from a monolayer to bulk solid/liquid or the collapse of monolayer took place, against temperature. Then, the rigidity of the monolayers or the modulus of monolayer elasticity (E), defined as the gradient of the π vs A isotherms, was also compared.

Energy levels of a cis-cyclopropane-containing and an α-methyl trans-cyclopropane-containing model molecule were calculated for both fully extended and U-shaped conformations. By using cis-cyclopropane-containing and α-methyl trans-cyclopropane-containing model molecules, computer simulation studies were performed to evaluate the bend-forming capacities of those groups.

**METHODS**

**Materials used for monolayer studies.** The samples used in the present study were MeO-MAs from M. kansasii 304, Mtb Canetti MNC1485, and Mtb H37Ra, and keto-MAs from MAC KK41-24 and Mtb H37Ra, prepared as described previously (Watanabe et al., 2001, 2002). These natural products all consist of a series of homologues. The ratios between cis- and trans-cyclopropane content of the MAs used and the details of their constituent homologues including their molecular weight content (%) and segment lengths, defined by the number of methylene groups n/m/l, which constitute the segment are given in Table 1. Relevant data, previously reported on the MeO- and keto-MAs from BCG and Mtb Aoyama B, are also included in Table 1, for reference.

**Other reagents.** Distilled reagent grade chloroform (Wako Chemicals) was used as the spreading medium. Water was distilled once and deionized with MilliQ Plus (resistance 18.2 MΩ cm).

**Surface pressure vs mean molecular area (π vs A) isotherm measurements.** The Langmuir monolayers were prepared by spreading a chloroform solution of MA (0.25–1 ml, approximately 10⁻³ M) on the water surface. Surface pressure (π) vs mean molecular area (A) isotherms of the Langmuir monolayer of MA were measured on a film balance, Lauda FW1 or FACE HBM700LB. The area of the water surface of the trough of the former was about 562 cm² and that of the latter 700 cm². The compression rate of the monolayer was 14 Å² molecule⁻¹ min⁻¹. The π vs A isotherms were measured at various temperatures in the range of 15–46 °C, and those of MA from Mtb H37Ra were measured at only two or three temperatures because of the scarcity of the materials available. The subphase temperature was controlled with an accuracy of ±0.2 °C. The room temperature was thermostatted at 23 ± 1 °C. Each measurement was repeated three to five times.

**Computer simulation studies on the bend-forming capacities of cis- and α-methyl trans-cyclopropane groups.**

**Energy calculations.** To evaluate the stability of the U-shaped conformation of cis- and α-methyl trans-cyclopropane-containing carbon chains, (1R,2S)-1,2-dibutyl-cis-cyclopropane and (1R,2S)-1-buty1-2-(R-pentane-2-yl)-trans-cyclopropane were chosen as model molecules. Then, simulating the conformation of the folded MAs, their U-shaped conformation models and also open-chain conformation models were prepared by assigning the carbons and surface pressures are very low (Villeneuve et al., 2010). However, the thermodynamic studies of the Langmuir monolayers show that different MAs have different conformational behaviours (Villeneuve et al., 2005, 2007, 2010). Thus, non-oxygenated α-MAs take W-form conformations only in a limited area where both temperatures and surface pressures are very low (Villeneuve et al., 2010). Oxygenated MAs, especially keto-MAs, generally form more stable W-form conformations partly owing to the likely hydration of the keto group on the water surface (Villeneuve et al., 2005, 2007). Our previous monolayer studies showed that the MeO-MAs and keto-MAs from Mycobacterium bovis Bacillus Calmette–Guérin (BCG) and Mycobacterium tuberculosis (Mtb) Aoyama B had distinct conformational behaviours, including different features of the surface pressure (π) vs mean molecular area (A) isotherms (Villeneuve et al., 2005, 2007). The constituent molecules of MeO-MAs and keto-MAs from BCG and those from Mtb are essentially the same, differing only in the ratios of the cis- and trans-cyclopropyl groups. The same applies to the keto-MAs from the two, suggesting a positive effect of the cis- or trans-cyclopropane configuration in the intra-chain group Y on the conformational behaviours (Villeneuve et al., 2005, 2007).

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Energy levels of a cis-cyclopropane-containing and an α-methyl trans-cyclopropane-containing model molecule were calculated for both fully extended and U-shaped conformations. By using cis-cyclopropane-containing and α-methyl trans-cyclopropane-containing model molecules, computer simulation studies were performed to evaluate the bend-forming capacities of those groups.
Computer simulations on small model molecules. The following small molecule models were used, whose methylene chains were of various torsion angles.

- \( \text{CH}_2(\text{CH}_3)\_\text{cis-cyclopropyl}-(\text{CH}_2)_n \text{-CH}_3 \) \((n=3, 4 \text{ and } 5)\)
- \( \text{CH}_2(\text{CH}_3)\_\text{trans-cyclopropyl}((\text{R},\text{S})-(\text{CH}_2)-(\text{CH}_2)_{n-1}) \text{-CH}_3 \) \((n=4 \text{ and } 5)\)

After energy minimization, those molecules were subjected to molecular dynamics (MD) [Sybyl 6.91, 1 pico second (ps)] and after each three 1 ps run, the distances between the third carbons from the cyclopropane ring were recorded. The number of the models subjected to the analysis was 68, 23 and 105 for \( \text{cis-cyclopropane} \)-containing small molecule models with \( n=3, 4 \text{ and } 5 \), respectively, and 62 and 25 for \( \text{trans-cyclopropane} \)-containing structures with \( n=4 \text{ and } 5 \), respectively.

**RESULTS**

**Monolayer studies**

The \( \pi \text{ vs } A \) isotherms of MeO-MA from *M. kansasii* were measured at various temperatures between 10 and 46 °C, of which representative traces are shown in Fig. 1(a). The filled and open arrowheads in the diagrams point at the corresponding phase transition points and film collapse points, respectively. The phase transition and the film collapse points are defined as described previously (Villeneuve et al., 2010). In the isotherms, multiple, often three transition points, \( \pi^{tr-1}, \pi^{tr-2} \text{ and } \pi^{tr-3} \) were noted. To construct the phase diagrams of the \( \pi \text{ vs } temperature \), \( \pi^{tr-1}, \pi^{tr-2} \text{ and } \pi^{tr-3} \) and film collapse point \( \pi^cP \) were plotted against \( T \) (Fig. 1b). To further characterize the phase transitions and film collapse observed, the molecular areas at the corresponding phase transitions \( A^{tr-1}, A^{tr-2} \text{ and } A^{tr-3} \) and at the collapse pressures \( A^cP \) were plotted against \( T \) (Fig. 1c). In Fig. 1(b), the characteristic features of an endothermic phase transition of MeO-MA, reported previously (Villeneuve et al., 2005, 2007), were noted, i.e. \( \pi^cP \) (filled symbols) decreased as the \( T \) increased. At the first phase transitions, \( \pi^{tr-1} \), the \( A^{tr-1} \) changed from approximately 80 Å² molecule⁻¹ to the \( A^cP \) values of 40–60 Å² molecule⁻¹.

In the \( \pi \text{ vs } A \) isotherms of the MeO-MA from Mtbc Canetti (Fig. 2), a bend is clearly observed at the collapse point of the monolayer \( (\pi^cP=31 \text{ mN m}^{-1}, A^cP=43 \text{ Å}^2 \text{ molecule}^{-1}) \) at 20 °C; \( \pi^cP=31 \text{ mN m}^{-1}, A^cP=40 \text{ Å}^2 \text{ molecule}^{-1} \) at 22 °C; \( \pi^cP=31 \text{ mN m}^{-1}, A^cP=38 \text{ Å}^2 \text{ molecule}^{-1} \) at 25 °C; \( \pi^cP=30 \text{ mN m}^{-1}, A^cP=38 \text{ Å}^2 \text{ molecule}^{-1} \) at 30 °C; and \( \pi^cP=30 \text{ mN m}^{-1}, A^cP=36 \text{ Å}^2 \text{ molecule}^{-1} \) at 37 °C. In addition to the film collapse point, kinks corresponding to phase transitions were noted at around \( \pi^{tr-2}=20 \text{ mN m}^{-1}, A^{tr-2}=54 \text{ Å}^2 \text{ molecule}^{-1} \) for 20 °C; \( \pi^{tr-2}=18 \text{ mN m}^{-1}, A^{tr-2}=53 \text{ Å}^2 \text{ molecule}^{-1} \) for 22 °C; \( \pi^{tr-2}=16 \text{ mN m}^{-1}, A^{tr-2}=53 \text{ Å}^2 \text{ molecule}^{-1} \) for 25 °C; \( \pi^{tr-2}=12 \text{ mN m}^{-1}, A^{tr-2}=55 \text{ Å}^2 \text{ molecule}^{-1} \) for 30 °C; and \( \pi^{tr-2}=5 \text{ mN m}^{-1}, A^{tr-2}=54 \text{ Å}^2 \text{ molecule}^{-1} \) for 37 °C. The dependence of those five \( \pi^{tr} \) values on \( T \) suggested an endothermic transition, characteristic of MeO-MA, i.e. phase transition from a W-form conformation to an extended one, though the \( A^{tr} \) value looked apparently too small for proper W-form to extended form phase transitions.

In the \( \pi \text{ vs } A \) isotherms of Mtbc H37Ra MeO-MA, shown in Fig. 3, no clear phase transition was observed; only the film collapse points were noted. At both of the temperatures, surface pressure remained almost at 0 mN m⁻¹ until the
monolayer was compressed to the mean molecular area of 60 Å² molecule⁻¹, when it started increasing smoothly up to 30 (25 °C) or 35 mN m⁻¹ (37 °C), where the monolayer collapsed.

The $\pi$ vs $A$ isotherms of keto-MAs from MAC, measured at different temperatures, are shown in Fig. 4(a). Its phase diagram $\pi_A$, $\pi_T$ vs $T$ and the mean molecular area $A^\pi$, $A^\rho$ vs $T$ diagram given in Fig. 4(b,c), respectively, had the common features of keto-MAs: the $A^\rho$ values were in the range of 75–80 Å² molecule⁻¹, corresponding to the area of MA in W-form conformation, and implying that the keto-MA retained the W-form conformation until the collapse pressure. Its $\pi^\rho$ values were about the same as those of the keto-MA from Mtba Ayama B reported previously (Villeneuve et al., 2005). There were phase transition-like kinks in the lower surface pressure region, which were interpreted as the results of minor conformational changes such as reorientation of the molecules in the monolayers, because, although the $\pi^\rho$ values of the kinks certainly decreased as $T$ increases, the degree of inclination or the enthalpy increment involved was too small for the conformational changes from a W-form conformation to an extended one.

The $\pi$ vs $A$ isotherms of keto-MAs from Mtba H37Ra (Fig. 5) measured at 10, 25 and 37 °C showed features quite different from those of other keto-MAs. The isotherm measured at 10 °C gave the $A^\pi$ at about 40 Å² molecule⁻¹ suggesting that the MA was in extended conformation when it collapsed. The phase-transition-like point observed ($\pi^\rho$=7 mN m⁻¹; $A^\rho$=55 Å² molecule⁻¹) was not likely a proper W-form to extended-form conformation transition, because its $A^\rho$ value was, at that surface tension, too small for that transition, which strongly suggested that at 10 °C the keto-MA from Mtba H37Ra did not take the W-form conformation. The $\pi$ vs $A$ isotherm at 25 °C, gave the collapse point of the monolayer at $\pi^\rho$=30 mN m⁻¹ ($A^\rho$=40 Å² molecule⁻¹) and no phase transition. The $\pi$ vs $A$ curve of 37 °C was slightly more complex: the $\pi^\rho$ value started increasing slowly from 0 mN m⁻¹ at $A$ ≈ 80 Å² molecule⁻¹, and after showing a shoulder-like transition kink ($\pi^\rho$ ≈ 2.5 mN m⁻¹; $A^\rho$ ≈ 75 Å² molecule⁻¹), the surface pressure started abruptly increasing at around $A$=50 Å² molecule⁻¹, from $\pi$=10 mN m⁻¹ to $\pi$=20 mN m⁻¹, where the first film collapse took place ($\pi^\rho$ ≈ 20 mN m⁻¹; $A^\rho$ ≈ 42 Å² molecule⁻¹). The second collapse occurred at $\pi^\rho$ ≈ 33 mN m⁻¹; $A^\rho$ ≈ 31 Å² molecule⁻¹.

To give a general view on the stability of the W-conformation, the $\pi^\rho$ values of MeO-MAs from M. kansasii and Mtba Canetti were plotted against $T$, along with the previously reported data on those from Mtba Ayama B and BCG for reference (Fig. 6). The figure demonstrated that all the $\pi^\rho$ values of MeO-MAs where the W-form conformation changed to extended form conformation decreased almost linearly as the $T$ increased. It also showed that the $\pi^\rho$ values of MeO-MA from Mtba Canetti (x-methyl trans-cyclopropyl content: 26 %) were more like those of MeO-MA from BCG (trans-cyclopropyl content: 6.4 %). A phase transition was noted in MeO-MA from Mtba Canetti at 37 °C, whereas it was not detected in that from BCG at temperatures above 34 °C. Of the three phase transitions $\pi^\rho$, $\pi^\rho$-2 and $\pi^\rho$-3 observed in the isotherms of MeO-MA from M. kansasii, the third phase transition, $\pi^\rho$-3,
behaved like that of MeO-MA from Mtb and the second one, πtrans-2, like that of MeO-MA from BCG.

The πtrans values of keto-MA from MAC at different temperatures were found to show the same film collapse features as those of keto-MA from Mtb and BCG, although not shown in a diagram here. The πtrans of keto-MA from Mtb H37Ra gave much lower values. The πtrans vs T curve of MAC keto-MA (trans-cyclopropyl content: 89%) lies in between those of Mtb Aoyama B (trans-cyclopropyl content: 73%) and BCG (trans-cyclopropyl content: 25%) in the temperature range of 10–30 °C and almost overlaps with that of Mtb Aoyama B at above T=30 °C. The πtrans values of keto-MA from Mtb H37Ra were definitely lower than those of the other MAs.

The modulus or the elasticity (E) of the MA monolayer was obtained by using the following equation:

$$E = -A \left( \frac{\partial \pi}{\partial A} \right)_{\tau}$$  \hspace{1cm} (1)

The E values of MeO-MAs in W-form conformation (generally at lower temperatures) (e.g. at π=15 mN m⁻¹, E=150–280 mN m⁻¹) were apparently higher than those in extended-form conformation (E=10–100 mN m⁻¹ at π=15 mN m⁻¹), and the E values of keto-MAs (E=200–480 mN m⁻¹ at π=15 mN m⁻¹, except for that from MAC) were very much higher than those of MeO-MAs in W-form conformation. Of the keto-MAs, that from MAC showed apparently much higher E values (E=580–2000 mN m⁻¹ at π=15 mN m⁻¹), which was also assayed in the relation to its z-methyl trans-cyclopropane containing MA content. The monolayer of keto-MA from MAC was found to be markedly stiffer by quite high E values than those of the other keto-MAs.

To show the relationship between the conformation of MAs in the monolayer and their trans-cyclopropane-containing MA content, the A values at π=15 mN m⁻¹ were plotted against the trans-cyclopropane-containing MA content (%) at 25 °C and at 37 °C, which are shown in Fig. 7(a, b), respectively.

Energy calculations and computer simulations

Energy calculations. The results of energy calculations showed that the energy level E of cis-cyclopropane-containing model molecule (1R,2S)-1,2-dibutyl-cis-cyclopropane of U-shaped conformation was −432.403 570 1 a.u. and that of open chain conformation, −432.406 464 4 a.u., whereas that of trans-cyclopropane-containing model molecule (1R,2S)-1-butyl-2-(R-pentane-2-yl)-trans-cyclopropane of the U-shaped conformation was −471.720 373 1 a.u. and that of open chain conformation, −471.718 917 9 a.u. Thus, for the cis-cyclopropane-containing molecule, the open chain conformation was shown to be energetically more stable than the U-shaped conformation by 1.82 kcal (7.61 kJ) mol⁻¹, and for the trans-cyclopropane-containing one, the U-shaped conformation to be more stable than the open chain conformation by 0.91 kcal (3.81 kJ) mol⁻¹.

Computer simulations on small model molecules. For the cis-cyclopropane-containing molecules with n=3, 4 and 5, the distances between the third carbons from the ring SD (Å) were 7.36 ± 0.61, 7.53 ± 0.40 and 7.45 ± 0.46, respectively. For the trans-cyclopropane-containing molecules with n=4 and 5, the corresponding distances SD (Å) were 6.34 ± 0.72 and 6.18 ± 0.45, respectively. The results showed that z-methyl trans-cyclopropane-containing molecules form
Our previous studies on the monolayers of the MeO- and tighter hairpin U-shapes than cis-cyclopropane-containing molecules.

**DISCUSSION**

Our previous studies on the monolayers of the MeO- and keto-MAs from Mtb Aoyama B and BCG (Villeneuve et al., 2005, 2007) suggested that their conformational behaviours were related to the nature of the intra-chain group Y. In the present study, to examine and analyse the effect of cis- and x-methyl trans-cyclopropane intra-chain groups on the conformation of MAs more precisely, monolayer features or phase diagrams of oxygenated MAs from Mtb Canetti, Mtb H37Ra, M. kansasi and MAC, having different cis-cyclopropane group and trans-cyclopropane group ratios, were assayed and the results were compared with those from MAs from Mtb and BCG reported previously (Villeneuve et al., 2005, 2007).

The major specific features observed in the isotherms may be summarized as follows. (a) MeO-MAs from M. kansasi showed the phase transition typical of MeO-MAs, with a change from W-form to extended-form conformation.

However, the isotherms gave plural, often three, phase transitions, implying that the monolayer was not behaving as a single homogeneous mass. (b) In MeO-MAs from Mtb Canetti, the $A^\alpha$ was much smaller than that expected for the transition from W-form to extended-form, though energetically the $A^\alpha$ was interpreted to be of the W-form to extended-form transition. (c) Keto-MAs from MAC showed typical keto-MA monolayer features, implying that they retained W-form conformation until the film collapsed. (d) MeO-MAs from Mtb H37Ra, whose trans-cyclopropane content is almost zero, did not show phase transition in their isotherms, and their $A^\alpha$ implied that when the monolayer collapsed, the MA was in extended-form conformation. However, in the case of x-MAs whose intra-chain groups are cis-cyclopropane, phase transition from W-form to extended form is clearly noted, demonstrating that they do take W-form conformation when the temperatures and surface pressures are lower (Villeneuve et al., 2010). (e) The isotherm of keto-MAs from Mtb H37Ra at 37 ºC showed two collapse points and one phase transition point, only a film collapse point at 25 ºC, and one film collapse point and a phase-transition-like kink at 10 ºC. Of the isotherms of MeO- and keto-MAs from Mtb H37Ra, only keto-MA at 37 ºC showed a proper phase transition point, which shows that keto-MA from Mtb H37Ra does take a W-form conformation, possibly owing to the stronger hydrogen bonding between the carbonyl group and the water surface.

In Fig. 7(a, b), showing the relations between the mean molecular area $A$ at $\pi=15$ mN m$^{-1}$ and x-methyl trans-cyclopropane-containing MA content at 25 and 37 ºC, respectively, a line can be drawn demonstrating that the A values are related, although very roughly, to the x-methyl trans-cyclopropane-containing MA content. Apparently, when the trans-cyclopropane-containing MA content was above 20–30 %, all of the MA molecules in the monolayer took W-form conformation. An analogous positive relationship was noted between the E values at $\pi=15$ mN m$^{-1}$ and x-methyl trans-cyclopropane-containing MA content. In Fig. 7(a, b), however, the A values of MeO-MAs from M. kansasi and Mtb Canetti were much lower than the A values expected from their x-methyl trans-cyclopropane content. This suggests that those MAs were behaving as if their actual trans-cyclopropane content was much lower than that implied by the H-NMR spectra. Fig. 6 showed that, of the three $\pi^\alpha$ values of MeO-MAs from M. kansasi, containing more x-methyl trans-cyclopropane groups than those from Mtb, the $\pi^\alpha-3$ values behaved like the $\pi^\alpha$ values of that from Mtb, and $\pi^\alpha+2$ more like that from BCG. Analogously, the $\pi^\alpha$ of MeO-MAs from Mtb Canetti behaved like those from BCG whose trans-cyclopropane content was very much less.

The following two facts may explain those observations (a–e) noted in the isotherms of monolayer studies, and the apparent difference between the trans-cyclopropane content obtained from the H-NMR analysis and that content implied by the results of Figs 6 and 7 for MeO-MA from

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**Fig. 7.** Mean molecular area vs x-methyl trans-cyclopropane-containing MA (%) at $\pi=15$ mN m$^{-1}$. (a) T, 25 ºC; (b) T, 37 ºC. M, MeO-MAs; K, keto-MAs. AoB refers to Mtb Aoyama B, Canetti to Mtb Canetti, kans to M. kansasi and H37Ra to Mtb H37Ra.
M. kansasii and Mtbc Canetti. A possible major cause may be the more complex overall composition of the relevant MAs. As shown in Table 1, in the samples of MeO-MAs from Mtb H37Ra, Mtbc Canetti, M. kansasii and of keto-MA from Mtb H37Ra, all showing atypical isotherm features, the sum of the three major components in each is about 50%, whereas that of other MAs showing typical isotherm features is above 70%. In other words, it may be stated that the number of the components needed to constitute over 70% of each sample is 5–6 for the former, whereas it is 2–3 for the latter. Perhaps in those more complex multi-component samples, not all of the components may form or behave as a homogeneous mixture, and some may form diverse domains that behave as independent phases. This assumption may be verified by the multiple phase transition points noted in the isotherms of MeO-MAs from M. kansasii, giving often three W-form to extended-form phase transition points, and by the W-form to extended-form conformation phase transition at an unexpectedly small A value by MeO-MAs from Mtbc Canetti, probably due to some components behaving differently. MeO- and keto-MAs from Mtb H37Ra each consist of two series of homologues, i.e. one cis-cyclopropane-containing and two cis-cyclopropane-containing ones in a ratio roughly of 1:1. Each homologue comprises a series of complex components (Watanabe et al., 2001). It is more unlikely for such a multi-component mixture to behave as a single homogeneous mass and if some components or partial domains in the mass undertake phase transitions, the others may obscure such transitions. Keto-MAs from MAC that form very solid compact W-form conformations (Fig. 7) and have extremely high E values (800–1200 mN m⁻¹ at π=15 mN m⁻¹) have only two components constituting over 80% of the sample (Table 1). The smaller A value than that from Mtb may be due to the more compact packing of the keto-MAs from MAC; the smaller amount of cis-cyclopropane-containing MA will help as such MAs require a larger area per molecule, even in W-form conformation.

The second reason is the variations in the segment lengths. Our previous paper on MD studies of α-MA conformations showed that differences in the m/l lengths with a cis-cyclopropane group in between affected the extended-form conformations of α-MAs (Villeneuve et al., 2010): when n/m/l was 15/14/19, after MD of the starting W-form conformation, most of the resulting MAs took completely extended-form conformation, whereas when it was 15/16/17, their extended conformation tended to include hairpin-like folding forms of l and m chains with a cyclopropane group in between, showing that when the segment lengths on both sides of the cis-cyclopropane ring were about the same, the folding conformation was to be more easily retained. The study also showed m/l of 14/17 and 16/17 had analogously stabilized or formed folded conformation, whereas 14/19 had scarcely any positive effect on retaining the folded conformation. Table 1 shows that the segment lengths on either side of cis-cyclopropane, n/m, in the MeO- and keto-MAs from BCG and Mtb are mostly 15/18, 17/16 or 17/18, whereas those in atypical isotherm features are often 19/12, 19/14, 21/12, 21/14 and 23/12. Thus, as suggested in our previous paper, larger differences between the lengths of the chains with a cis-cyclopropane ring in between may more actively accelerate the relevant segments to expand, thus compensating for the folding effect of the α-methyl trans-cyclopropane group. Thus, the deviation noted in Figs 6 and 7, apparently contradictory to the present theory that α-methyl trans-cyclopropane content is directly related to the rate of W-form conformation of MA and that the group takes an active role in deciding the folding conformation of MAs, is thus properly accounted for.

The energy calculation and the MD simulation results positively support the conclusion derived from the present monolayer studies that the α-methyl trans-cyclopropane ring plays a positive role in deciding the conformation of MAs or contributes to formation of tighter solid bends than the cis-cyclopropane ring. It was particularly striking that the folded U-shaped conformation of the α-methyl trans-cyclopropane-containing model compounds were energetically preferred to the open chain form. This demonstrates the inherent value of these α-methyl trans-cyclopropanes as excellent articulation units in long fatty acid chains to promote very efficient folding. It should be noted here that the presence of the methyl branch adjacent to the methoxy and keto units in MeO- and keto-MAs is also most probably a key factor in allowing these MAs to fold efficiently at the distal position (X) in meromycolates.

The anomalous results for Mtb H37Ra are not unexpected as this organism is a stable attenuated variant of the virulent type strain Mtb H37Rv. The lack of virulence may be related to the inability of the unusual oxygenated MAs to adopt the usual conformations typical of virulent strains, such as Mtb Aoyama B (Villeneuve et al., 2005). Some extractable complex lipids, such as sulfolipids and acyl trehalose, are also perturbed in Mtb H37Ra, in comparison with Mtb H37Rv (Minnikin et al., 1986). However, Mtb H37Ra is a viable organism, so the presence of unusual oxygenated mycolic acids, rich in cis-cyclopropane, still allows efficient bacterial growth in vitro.

The importance of oxygenated MAs in the virulence of Mtb has been indicated in a number of studies. A key role for keto-MAs in growth in host cells was indicated by Yuan et al. (1998) and oxygenated MAs were necessary for virulence in mice (Dubnau et al., 2000). More specifically, it was found that the inflammatory activity of trehalose dimycolates (TDMs) was suppressed by trans-cyclopropagation of the constituent MAs (Rao et al., 2006). In a parallel study, it was found that trehalose dimycolates, rich in keto-MAs, formed large, stable micelles whose toxicity in mice was reduced in comparison with TDMs with other MA classes (Fujita et al., 2007). The inhibition of the cyclopropagation of oxygenated MAs resulted in cell death of Mtb and increased susceptibility to antibiotics (Barkan et al., 2009). Synthetic cis-cyclopropyl MeO-MAs and keto-MAs were inflammatory, but α-methyl trans-cyclopropyl MeO-MAs showed reduced activity and α-methyl trans-cyclopropyl keto-MAs
were non-inflammatory (Vander Beken et al., 2011). In a recent study, pellicle growth of Mtb was shown to have an absolute requirement for keto-MAs (Sambandan et al., 2013). A general conclusion from these studies is that keto-MAs, in particular, are essential for overall pathogenicity of Mtb, but their relatively reduced direct biological activity indicates a more passive structural role facilitated by their efficient folding and packing, resulting from the presence of the \( \alpha \)-methyl \( \text{trans} \)-cyclopropane units.

Naturally occurring mycobacterial MAs are found as highly complex mixtures, presumably adapted to provide a suitable lipophilic environment in the cell envelope. It is necessary to determine the importance of particular structural features in these molecules and this study has focused on certain oxygenated MAs, which include either \( \text{cis} \)- or \( \text{trans} \)-cyclopropane rings. There is a lack of systematic physical studies on simple \( \text{cis} \)- or \( \text{trans} \)-cyclopropane fatty acids, but the situation is similar to \( \text{cis} \)- or \( \text{trans} \)-olefinic fatty acids, as indicated in a differential thermal analysis study on phosphatidylcholines incorporating analogous \( \text{cis} \)- or \( \text{trans} \)-olefinic and cyclopropane acyl chains (Silvius & McElhaney, 1979). These studies showed that fatty acid fluidity increased in the order \( \text{cis} \)-olefin < \( \text{trans} \)-cyclopropane < \( \text{trans} \)-olefin < \( \text{cis} \)-cyclopropane < \( \text{cis} \)-olefin. Certain mycobacteria, such as the more rapidly growing \textit{Mycobacterium marinum} (Daffe\' et al., 1991), have MeO- and keto-MAs with both \( \text{cis} \)-olefinic and \( \alpha \)-methyl \( \text{trans} \)-olefinic intra-chain Y groups. The above trend leads to the conclusion that the cycloproplyl MAs, in members of the Mtb complex, will produce a more condensed permeability cell envelope barrier than the olefinic MAs from \textit{M. marinum}.

It is well-established that monolayers of \( \text{cis} \)-\( \Delta \)-9-octadecenoic acid (oleic acid) are more expanded than those from \( \text{trans} \)-\( \Delta \)-9-octadecenoic acid (elaidic acid), which in turn are much less condensed than octadecanoic acid (stearic acid) (Welles et al., 1975). This general principle was confirmed by more recent studies on \( \text{cis} \)- and \( \text{trans} \)-\( \Delta \)-13-docosenoic acids (Vollhardt, 2007). It is logical to assume, therefore, that a mid-chain \( \text{cis} \)-cyclopropane ring would result in relatively more expanded monolayers than those for a corresponding fatty acid with an isolated \( \text{trans} \)-cycloproplyl unit. Similarly, a simple methyl branched fatty acid, such as tuberculostearic (10-methyl octadecanoic) acid, can be predicted to be more expanded than straight-chain octadecanoic acid. Consequently, an \( \alpha \)-methyl branch, adjacent to the \( \text{trans} \)-cycloproplyl unit in oxygenated MAs, would be expected to increase the flexibility of fatty acids incorporating a relatively rigid isolated \( \text{trans} \)-cyclopropane ring.

The role of oxygenated \( \text{trans} \)-cycloproplyl MAs in influencing the fluidity of the mycobacterial cell wall has been studied in detail (Liu et al., 1996). It was found that the proportion of \( \text{trans} \)-cycloproplyl MAs increased in \textit{M. avium} cultivated at higher growth temperatures. Similarly, thermal transitions in intact cells were higher when elevated proportions of \( \text{trans} \)-cycloproplyl MAs were present. These findings were interpreted as being due to ‘less disruptive \( \text{trans} \) structures’ raising the melting temperature of the cell wall and reducing fluidity (Liu et al., 1996). It was also stated that ‘\( \text{trans} \) structures, unlike \( \text{cis} \) structures, are not expected to disturb the lateral packing of hydrocarbon chains as severely’. This was presumably based on the premise that simple \( \text{trans} \)-cycloproplyl fatty acids pack together more closely than the corresponding \( \text{cis} \)-cycloproplyl acids. The true explanation, revealed in the current paper, is that tight packing of \( \text{trans} \)-rich MAs is actually due to facile folding to produce close-packed W-form conformations, which would result in the observed higher thermal transitions. In essence, the \( \alpha \)-methyl branch transforms a relatively rigid isolated \( \text{trans} \)-cyclopropane ring into a flexible articulated unit ideally suited for enhanced folding of mycobacterial MAs.

**CONCLUSION**

The importance of the \( \alpha \)-methyl \( \text{trans} \)-cyclopropane unit in facilitating efficient folding of the oxygenated MAs from Mtb has been demonstrated. This key structural unit allows keto-MAs, in particular, to assume tight W-form conformations with all four hydrocarbon chains in parallel. This characteristic behaviour of the \( \alpha \)-methyl \( \text{trans} \)-cyclopropane unit was supported by energy calculations and the MD simulations on model compounds. The behaviour of representative MeO- and keto-MAs in monolayer experiments showed positive correlation between \( \text{trans} \)-cyclopropane content and fully folded W-form conformations. It was also found that oxygenated MAs form more coherent monolayers when there are a smaller number of homologues in the natural acids. The unusual MA content of the attenuated variant Mtb H37Ra resulted in unstable W-form conformations in the Langmuir monolayer.

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