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Structure determination of lipopeptides from *Mycobacterium avium* subspecies *paratuberculosis* and identification of antigenic lipopeptide probes

Katsuhiko Mitachi¹, Lekh Nath Sharma Gautam¹, Jeffrey H. Rice², Keiko Eda², Ashutosh Wadhwa², Eiichi Momotani³, Joseph P. Hlopak², Shigetoshi Eda², and Michio Kurosu¹

Michio Kurosu: mkurosu@uthsc.edu

¹Department of Pharmaceutical Sciences, College of Pharmacy, University of Tennessee Health Science Center, 881 Madison Avenue, Memphis, TN 38163-0001, United States

²Center for Wildlife Health, Department of Forestry, Wildlife and Fisheries, University of Tennessee Knoxville, 274 Ellington Plant Science, Knoxville, TN 37996-4563, United States

³Tohto College of Health Sciences, Department of Human-care, 4-2-11, Kamishibamachi, Nishi Fukaya, Saitama 366-0052, Japan

Abstract

Mycobacterium avium subsp. *paratuberculosis* (MAP) causes chronic illnesses mostly in ruminants. MAP infection of intestinal tissue triggers a fatal inflammatory disorder, Johne's disease (Paratuberculosis). Development of fast and reliable diagnostic methods for Johne's disease in clinically suspected ruminants requires the discovery of MAP-specific antigens that induce immune responses. Despite a long-time interest in finding such antigens that can detect serum antibody responses with high sensitivity, the antigens currently used for a diagnosis of the MAP infections are the crude extracts from the whole cell. We performed the serum antibody response assay-guided purification of the ethanol extract from MAP isolated from an infected cow. As the results of extensive fractionations and *in vitro* assays, we identified that arachidil-D-Phe-N-Me-L-Val-L-Ile-L-Phe-L-Ala-OH (named lipopeptide II β , 3) exhibited the highest antibody binding activity in serum of a MAP-infected cattle compared to the other lipopeptides isolated from MAP. The absolute chemistry of 3 was determined unequivocally via our HPLC-amino acid data bases. α -Amino lipopeptide II β and its fluorescent probes were synthesized and evaluated in serum antibody binding activity assays. Lipopeptide II β -(2*S*)-NH₂ (9) and its dansyl and FITC probes (10 and 11) exhibited antibody-mediate binding activity, and thus, such MAP specific lipopeptide probes can be potential biomarkers for the development of rapid and accurate diagnosis of Johne's disease.

Address correspondence to Michio Kurosu, mkurosu@uthsc.edu.

Supplementary Information

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Keywords

Lipopeptide; New chiral reprivatizing agents; *Mycobacterium avium* subspecies *paratuberculosis*; Johne's disease; Lipopeptide probes

Mycobacterium avium subsp. *paratuberculosis* (MAP) causes life-threatening chronic illnesses in animals.¹⁻⁴ Unlike other *Mycobacterium spp.*, MAP can adapt to gastrointestinal tract of ruminants, and its infections trigger a chronic inflammatory disorder, Johne's disease (Paratuberculosis).^{5,6} Particularly, bovine Johne's disease causes serious economic impact in the U.S. and other countries.⁷⁻¹⁰ Because neither practical treatment nor effective vaccine is available for bovine Johne's disease, rapid and accurate diagnostic methods are very important to confirm the diseases.¹¹⁻¹³ The enzyme-linked immunosorbent assay (ELISA) for detection of serum antibody is the most rapid and least costly test.¹⁴⁻¹⁶ However, antigens used for this assay are the crude mixtures derived from bacterial whole-cells that may cause false- positive and -negative results. Development of early diagnosis of MAP infections has been hampered by lack of studies on MAP-specific antigens.¹¹ Eckstein et. al. reported that Para-LP-01 (Fig. 5) is a major lipopeptide, which was isolated as a mixture of fatty acids.¹⁷ Para-LP-01 is the only known lipopeptide isolated from MAP to date. Unfortunately, in our studies it was concluded that Para-LP-01 did not show strong antibody response and cell-mediated immune response binding activities at a practical concentration (10 µg/mL). Therefore, we aimed to identify a new and more sensitive MAP-specific diagnostic antigen for Johne's disease ELISA tests. We previously reported that EtOH extract of MAP could be used for the detection of anti-MAP antibodies in serum and milk of MAP infected cattle, indicating that antigenic small molecules should exist in the EtOH extract.¹⁹ Such molecules could also be promising molecules to study humoral immunity in Johne's disease.^{11,20}

Materials and methods

Chemical materials and methods

Difco Middlebrook 7H10 agar, Middlebrook 7H9 broth, Tryptic soy agar, Tryptic soy broth, MOPS, tris(hydroxymethyl)aminomethane, 2-mercaptoethanol, sucrose and triton-X 100 were purchased from Sigma-Aldrich. ADC enrichment was purchased from Fisher Scientific. Magnesium chloride and potassium chloride were obtained from VWR. All reagents and solvents were commercial grade and were used as received without further purification unless otherwise noted. Flash chromatography was performed with Whatman silica gel (Purasil 60 Å, 230–400 Mesh). Analytical thin-layer chromatography was performed with 0.25 mm coated commercial silica gel plates (EMD, Silica Gel 60F₂₅₄) visualizing at 254 nm, or developed with ceric ammonium molybdate or anisaldehyde solutions by heating on a hot plate. ¹H-NMR spectral data were obtained using 400, and 500 MHz instruments. ¹³C-NMR spectral data were obtained using 100 and 125 MHz instruments. For all NMR spectra, δ values are given in ppm and *J* values in Hz.

Synthesis and chemical characterizations of lipopeptide II β (3) and its fluorescent probes, 11 and all new lipopeptide analogs studied in this article are summarized in supporting information.[†]

Serum samples

Four serum samples used in this study were kindly provided by Dr. Randy Capsel at the United States Department of Agriculture/National Veterinary Services Laboratory. Two Johne's disease (JD)-negative serum samples (ID 15 and 22) were collected from female Holstein cattle (3.3 and 5.9 years old, respectively) in a Minnesota dairy farm where no Johne's disease has been reported. Two JD-positive samples (ID 238 and 242) were collected from female Holstein cattle (4.0 and 5.0 years old, respectively) that were tested positive for Johne's disease by a commercial enzyme-linked immunosorbent assay (ELISA) (IDEXX Laboratories, Westbrook, MA) and a standard fecal culture test.

Preparation of ethanol extract of *Mycobacterium avium* subsp. *paratuberculosis* (MAP)

MAP strain (Linda) used for the EVELISA antigen preparation were obtained from the Agricultural Research Service, U.S. Department of Agriculture (Ames, Iowa). MAP organisms were cultured in Middlebrook's medium (Becton Dickinson, Cockeysville, MD) with the addition of 0.05% Tween 80 (Fisher Scientific, Fair Lawn, NJ) and 2 mg/l mycobactin J (Allied Monitor, Fayette, MO) at 37°C until used for experimentation. Ethanol extract of MAP was prepared as described previously. Briefly, bacilli of MAP were harvested from the liquid culture, suspended in 80% ethanol, and agitated by vortex to dislodge surface antigens. After removing MAP bacilli by centrifugation, ethanol extract was dried under vacuum.

Thin layer chromatography (TLC) of ethanol extract of *Mycobacterium* spp

MAP strains (K10 and Linda) were cultured and ethanol extract was prepared as described above. *M. gordonae* (TMC 1324), *M. phlei* (TMC 1548), *M. smegmatis* (TMC 1546), *M. terrae* (TMC 1450), *M. avium* subsp. *avium* (TMC 706, TMC 724), *M. bovis* BCG (TMC 1011), *M. flavescens* (TMC 1541), *M. fortuitum* (TMC 1529), *M. intracellulae* (TMC 1406), *M. kansasii* (TMC 1204), *M. scrofulaceum* (TMC 1323), and *M. szulgai* (TMC 1328) by Dr. Pamela Small (the University of Tennessee Knoxville). These are cultured as described above for MAP except that the medium did not contain mycobactin J or the conditions provided by ATCC. Bacilli were harvested from liquid cultures at stationary phase and centrifuged at 5,000×g for 10 min. The pellet (~80 mg) was resuspended in 1 mL of 80% EtOH, and agitated by vortex at room temperature for 1 min., followed by centrifugation at 10,621 × g for 10 min. The resulting supernatant was filtered through a 0.22µm filter. The 1 mL of 80% EtOH extracted antigens was dried and further purified using a variation of the Folch Wash Method, resuspended in 1 mL of CHCl₃ : MeOH (2:1), and rocked for two hours. The Folch extraction was performed by the addition of 0.2 volumes of water, rocked for 1 h and centrifuged at 10,621 × g for 10 min. The CHCl₃ layer was concentrated by drying overnight in fume hood. For one dimensional TLC, the chloroform fraction was dissolved in 1.6 mL of chloroform and 10 µl of the solution was loaded onto aluminum-backed silica-gel-60 plates (Merck), then developed with CHCl₃-MeOH-H₂O (150:10:1). Developed plates were sprayed to detect general components with Ce(SO₄)₂/((NH₄)₂MoO₄) in 2 M

sulfuric acid and charred. For two dimensional TLC, 10 µg dried weight of the chloroform layer dissolve in 10 µl of CHCl₃ was loaded onto the silica gel and developed with CHCl₃ : MeOH (94 : 6) for the first dimension and then with toluene : acetone (80 : 20) for the second dimension followed by staining with CuSO₄ (3%), H₃PO₄ (15%) in H₂O.

Detection of antibody binding to MAP antigens

Antibody binding to purified and chemically synthesized MAP antigens used in this study was conducted as described previously. MAP antigens were solubilized into dimethylsulfoxide (DMSO) at the concentration of 10 mg/ml and stored at -20°C until use. The stock solution of MAP antigen was diluted to desired concentration (e.g. 10 µg/mL) with absolute ethanol. The diluted MAP antigen solution was inoculated into a well of a 96-well plate (medium binding, Corning, NY) by evaporating ethanol, reacted with diluted (1:100) serum samples followed by horseradish peroxidase-labeled goat anti-bovine IgG (H +L) polyclonal antibody (diluted 1:500, Jackson ImmunoResearch Lab, Westgroup, PA). For dilution of serum sample and the secondary antibody, 10 mM phosphate buffered saline (pH 7.0) containing 0.05% Tween 20 and 10% SuperBlock (Thermo Scientific, Rockford, IL) was used. Binding of serum antibody was detected by using a substrate of horseradish peroxidase (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) according to the manufacturer's instructions.

Results and discussion

A number of bacterial species produce lipopeptides.²¹ Most of the antigenic lipids of *Mycobacterium subspecies* contain sugar moieties (e.g. 2,3-diacyl trehalose, phenolic glycolipids, lipooligosaccharides, and trehalose dimycolate).^{17,22} Interestingly, the lipid profile of MAP was quite different from that of the other *Mycobacterium spp.* (Fig. 1A). MAP is the closest species to *M. avium* subsp. *avium*, however, the lipid profile of MAP was significantly different from that of *M. avium* subsp. *avium* (lanes 5–6 vs 7–8 in Fig. 1A). As shown in Fig. 1B, presence of MAP-specific lipopeptides was confirmed by two-dimensional TLC; two major spots (indicated by circle) were found to be specific to MAP. The most abundant lipids found in MAP are lipopeptides (indicated by arrow in Fig. 1B). In our and other group's analyses of MAP lipopeptides, it was revealed that they often contain the *N*-methyl α-amino acid(s).¹⁷ Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide, FDAA), which is commonly used for the chiral analysis of amino acids, reacts with *primary* amines and their derivatives are analysed by HPLC.²³ Generally, FDAA showed the good enantioselectivity, but some amino acids (e.g. Tyr) and *N*-methyl amino acids showed poor reactivity against Marfey's reagent.²⁴ We have developed an unprecedented chiral derivatizing agent **5** (Fig. 3) that allows for the determination of the absolute configurations of a wide range of amino acids including *secondary* amines by ¹H-NMR or HPLC.²⁵ We have generated universal ¹H-NMR and HPLC data bases of α-amino acids and their *N*-methyl derivatives in order to determine relative and absolute stereochemistry of the amino acid components of MAP lipopeptides. In the present work, we report a general method to determine the structure of lipopeptides and new MAP lipopeptides, named lipopeptide IIα-γ (2–4) and MAP lipopeptide probes **9–11** that showed the serum antibody response via ELISA.

Isolations of antigenic lipopeptides

In an effort to identify antigenic molecules (elicitors) in the EtOH extract of MAP, we cultured MAP and obtained 50g of bacterial cell pellets. The MAP EtOH extract was subjected to a set of fractionation processes that were guided by the serum antibody response tests using an ELISA-based method. In the ELISA using sera prepared from a MAP-negative and -positive cattle, it was realized that the antigenic molecules are included in the fraction 4 (Fr4 in Fig. 2A). The Fr3 and Fr4 were determined to be lipopeptide-containing fractions based on a lipid-specific stain and ^1H -NMR analyses. The molecule containing in the Fr3 was the most abundant lipopeptide (~0.2% isolation yield from 50g of cell pellets), named lipopeptide I (**1**). The Fr3 and purified **1** did not exhibit antibody response at a 10 $\mu\text{g/mL}$ concentration.¹¹ Lipopeptide I (**1**) was structurally identical to Para-LP-01¹⁰ except for that **1** was a mixture of saturated fatty acids (stearic acid ($\text{C}_{18:0}$) : eicosanoic acid ($\text{C}_{20:0}$): docosanoic acid ($\text{C}_{22:0}$) = 1.0 : 2.3 : 1.38), whereas, Para-LP-01 was reported as a mixture of $\text{C}_{16:0}$, $\text{C}_{17:0}$, $\text{C}_{18:0}$, $\text{C}_{19:0}$, $\text{C}_{20:0}$, $\text{C}_{21:0}$, and $\text{C}_{22:0}$. The antigenic lipopeptide fraction (Fr4 in Fig. 2A) showed very poor solubility in CDCl_3 , DMSO-d_6 , THF- d_8 , and D_2O , but dissolved well in CD_3OD , albeit, lipopeptide I dissolved in a wide range of NMR solvents. Due to poor solubility of Fr4 in conventional organic solvents for silica gel chromatography, further purification required applying reverse-phase HPLC. Under the HPLC conditions (solvent system: MeOH : 0.1% TFA in H_2O = 95 : 5 to 100 : 0) Fr4 was purified and its chromatogram is illustrated in Fig. 2B. Among the fractionated samples, Fr4-4, Fr4-5, and Fr4-6 were isolated as their pure forms [named lipopeptide $\text{II}\alpha$ - γ (**2–4**)] with quantities of 5.9, 13.3, 6.1 mg, respectively. All fractions (Fr4-1 to Fr4-7) obtained via HPLC purification were evaluated in ELISA for determining their serum antibody responses. At 10 $\mu\text{g/mL}$ concentration, the HPLC fractions (Fr4-1~3 and Fr4-7 in Fig. 2B) did not show a significant activity against serum antibodies in a MAP-infected cattle compared with that of the negative control (DMSO). On the other hand, lipopeptide $\text{II}\alpha$ - γ (Fr4-4, Fr4-5, and Fr4-6) exhibited serum antibody binding activities; serum antibody binding against these fractions were significantly higher than those against the other fractions.

Structure determinations of lipopeptide $\text{II}\alpha$, $\text{II}\beta$, and $\text{II}\gamma$

Eckstein and co-workers determined the gross structure of Para-LP-01 via a combination of standard analytical methods including esterifications of the hydrolyzed amino acids with (*R*)- and (*S*)-2-butanol to determine their absolute stereochemistries.¹⁷ Esterifications of *N*-unprotected amino acids generally resulted in low conversions and their purification/analyses are time-consuming processes. Disadvantages of Marfey's reagent for *N*-methyl amino acids and less nucleophilic amino acids are discussed above. In order to systematically determine structure of MAP lipopeptides including their absolute stereochemistry in a single chemical modification followed by HPLC analysis, we have generated universal HPLC data bases for a series of carbamates, which were synthesized via coupling reactions between natural and unnatural amino acids and chiral derivatizing agent (*S*)-**5** and (*R*)-**5**.^{26,27} The HPLC data base of the carbamates **6** was realized as a powerful asset for determination of relative and absolute structures of minute quantities of α -amino acids and their *N*-methyl derivatives. As shown in Fig. 4B, all diastereomers derived from L-

and D-series did not show problematic peak overlaps, and thus, the simultaneous determinations of relative/absolute configurations of α -amino acids were realized. Usefulness of the HPLC database of the α -amino acid-carbamates were demonstrated by using the crude mixture of amino acids derived from lipopeptide (**1**) via hydrolysis with 6N HCl for 6h at 105 °C. The carbamate formations of the hydrolyzed amino acids under $^i\text{Pr}_2\text{NEt}$ in acetone/ H_2O (3/1) furnished the corresponding products in quantitative yields (Fig. 3A). The generated carbamates showed excellent resolution on a C_{18} -column using a mixture of solvents (AcOH and NaOAc in H_2O -MeOH-MeCN) (Fig. 3B). Comparison of HPLC retention times of **6a–h** with those of the database revealed that lipopeptide I (**1**) was determined to contain L-Ala, L-Phe, L-Ile, *N*-Me-L-Val, and D-Phe as observed for Para-LP-01. The fatty acid moiety of **1** was determined to exist as a mixture of C_{18} -, C_{20} -, and C_{22} -saturated fatty acids via GC/MS analyses of the trimethylsilyl (TMS)-esters of the acid-hydrolysis product. NOESY experiments of **1** in CD_3OD revealed that the *N*-methyl protons of *N*-Me-L-Val exhibited strong correlations with D-Phe and L-Ile residues, and the α -protons of the fatty acid moiety. Similarly, the *N*-Me-L-Val residue showed correlations with L-Ile and the partial structure of L-Phe-L-Ala-OMe was confirmed via NOESY correlations as illustrated in Fig. 4. Thus, the gross structures of lipopeptide I was unambiguously determined by single chemical transformations of the acid-hydrolysis product with (*S*)-**5** followed by its HPLC analysis, fatty acid, and 2D-NMR analyses.

Amino acid analyses of the acid-hydrolysis products of lipopeptide $\text{II}\alpha$ (**2**), $\text{II}\beta$ (**3**), and $\text{II}\gamma$ (**4**) realized that their peptide portions also consist of L-Ala, L-Phe, L-Ile, *N*-Me-L-Val, and D-Phe (Fig 3B and 3C), however, ^1H -NMR data for **2**, **3**, and **4** indicated that their *C*-terminal ends were not esterified with the methyl group. NOESY experiments and fatty acid analyses by GC-MS suggested the gross structures of lipopeptide $\text{II}\alpha$, $\text{II}\beta$, and $\text{II}\gamma$ as shown in Scheme 1. The pentapeptide moiety of **2**, **3**, and **4** were the same peptide sequence of D-Phe-L-(*N*-Me)Val-L-Ile-L-Phe-L-Ala-OH as observed in **1**. Interestingly, their structures were differ in the lipid moiety; **2**, **3**, and **4** were the *N*-acyl pentapeptide of pure stearic acid ($\text{C}_{18:0}$), arachidic acid ($\text{C}_{20:0}$), and behenic acid ($\text{C}_{22:0}$), respectively. In order to confirm relative and absolute stereochemistry of a series of lipopeptide II, **3** was chemically transformed to lipopeptide I-*N*-arachidic form. Treatment of **3** with CH_3OH under a mild esterification condition (EDCI, NaHCO_3 , Glyceroacetone-Oxyma in 5% H_2O - CH_3CN) furnished the corresponding methyl ester **7** in greater than 95% yield.^{28–30} The synthetic **7** was determined to be identical to the sample of natural **1** by ^1H -NMR, ^{13}C -NMR, $[\alpha]_D$, and TLC, except for the number of methylene protons and carbons in ^1H -NMR and ^{13}C -NMR, respectively. Consequently, gross structures of lipopeptide $\text{II}\alpha$ (**2**), $\text{II}\beta$ (**3**), and $\text{II}\gamma$ (**4**) were unequivocally determined.

Design and synthesis of the lipopeptide II probe

Lipopeptide I (**1**) and Para-LP-01 did not exhibit serum antibody binding activity at a 10 $\mu\text{g}/\text{mL}$ concentration.¹⁸ Similarly, the *C*-terminal-capped derivatives **7** and **8** synthesized by methylation or amide-formation of lipopeptide $\text{II}\beta$ (**3**) (see supporting information) quenched the serum antibody binding activity of **3**,²⁸ which were determined via ELISA (Scheme 1 and Fig. 6). Thus, we concluded that the *C*-terminal end of the lipopeptide requires being the free carboxylic acid to be an antigenic species. Based on the NOESY data

and lowest energy conformers obtained *via* grid-Monte Carlo conformational search algorithm,³¹ one of the most plausible conformers of lipopeptide II β (**3**) was speculated as shown in Fig. 5. It was hypothesized that functionalization of the 2 α -position (indicated by arrow in Fig. 5) of the lipid chain may not change the overall conformation of the pentapeptide portion, retaining a specific serum antibody response activity. We designed (2*S*)-aminolipopeptide II β (**9**) and its fluorescent probes **10** and **11** (Scheme 2). The synthesis of the pentapeptide was conducted via water-mediated peptide-coupling conditions. The pentapeptide **12** was synthesized by using Glyceroacetone-Oxyma, EDCI, and NaHCO₃ in water without formation of racemization products.²⁵ A simple aqueous NaHCO₃ work-up could remove all reagents utilized in the reactions to afford coupling products in over 85% yield for 8 steps; sequential couplings of the building blocks and deprotections of the Boc group in solution phase furnished five gram quantities of the pentapeptide **12**. The Boc protected (2*S*)-aminoarachidic acid **15** was synthesized via the Corey's chiral phase transfer catalyst (PTC)^{32,33}; asymmetric α -alkylation of the O'Donnell's glycine ester **13** at 0 °C provided **14** in 90% yield with the selectivity of 9/1.³⁴ Hydrolysis of the benzophenone-Schiff base and protection of the free amine with Boc₂O gave rise to **15** in >95% overall yield. Coupling between **12** and **15** under Glyceroacetone-Oxyma, EDCI, and NaHCO₃ in a mixture of DMF and water (10/1) followed by global deprotections provided (2*S*)-aminolipopeptide II β (**9**) in >85% overall yield. The fluorescent probes, **10** and **11** were synthesized via coupling reactions with the corresponding isothiocyanates. The crude products were purified via reverse-phase HPLC to furnish **10** and **11** in 95–100% yields.

Specific serum antibody responses of lipid II β fluorescent probes

In vitro assays of 2 α -amino lipopeptide II β (**9**) revealed that relative antibody binding activity of lipopeptide II β (**3**) was not significantly reduced by the introduction of the amino group at the 2 α -position of the lipid moiety (Fig. 6). Each compound was screened in three times of more on different days. Gratifyingly, it was realized that the two fluorescent derivatives of **10** and **11** also exhibited serum antibody binding activity in ELISA.

Discussions and Conclusions

In conclusion, the HPLC database of the α -amino acid-carbamates derived from (*S*)-**5** are demonstrated to be a convenient asset for structure determination of amino acids and their absolute configurations simultaneously. Amino acid analyses of lipopeptide I (**1**) and II α - γ (**2–4**) can be performed via our HPLC database that allows determining their gross structures in combinations with ¹H-NMR and GC-MS. The method using the chiral derivatizing agents **5** has a significant advantage over currently available methods for determination of the absolute configuration of free amino acids via ¹H-NMR spectroscopy and/or HPLC.²⁵

Lipopeptide I (**1**), a methyl ester, did not exhibit humoral immune activity against serum antibodies in MAP-infected cattle at the concentrations tested. On the contrary, the free carboxylic acid, lipopeptide II α - γ (**2–4**) showed serum antibody response activities in the same assay systems (the data only for **3** is shown in Fig. 6). Lipopeptide II α - γ are

isolated in a minute quantity, whereas, lipopeptide I contains ~0.2% in wet MAP cell pellets. Culturing a slow-growing MAP and purification of large quantity of lipopeptide I for the generation of lipopeptide II β require significant efforts and time. Thus, enough antigenic lipopeptide II β (**3**) is secured via a chemical synthesis; a gram quantity of **3** is achieved via water benign peptide-forming reactions in solution phase. Importantly, an antigenic lipopeptide II β (**3**) can be modified at the C2'-position of the lipid moiety. The free amino group of lipopeptide II β -(2*S*)-NH₂ (**9**) will be applied onto polymer supports (e.g. poly-D-lysine coated well plates) for development of a reliable serum antibody ELISA. Lipopeptide II β -(2*S*)-NH-FITC (**11**) will be an ideal probe for a fluorescence polarization (FP) assay (excitation; 480 nm emission; 535 nm) of serum samples from MAP-infected cattle. Application of new lipopeptide probes described here for accurate diagnosis of Johne's disease will be reported elsewhere.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A. Comparison of lipids in EtOH extract of *Mycobacterium* spp. Spots specific to MAP are indicated by arrows. Lane 1. *M. goodii* (TMC 1324), 2. *M. phlei* (TMC 1548), 3. *M. smegmatis* (TMC 1546), 4. *M. terrae* (TMC 1450), 5. MAP (K10), 6. MAP (Linda), 7. *M. avium* subsp. *avium* (TMC 706), 8. *M. avium* subsp. *avium* (TMC 724), 9. *M. bovis* BCG (TMC 1011), 10. *M. flavescens* (TMC 1541), 11. *M. fortuitum* (TMC 1529), 12. *M. intracellulae* (TMC 1406), 13. *M. kansasii* (TMC 1204), 14. *M. scrofulaceum* (TMC 1323), 15. *M. szulgai* (TMC 1328). **B.** Two-dimensional TLC analysis of lipid components in EtOH extract of MAP and *M. avium* subsp. *avium*. Spots specific to MAP are indicated by circles. TLC conditions: A. CHCl_3 : MeOH : H_2O (150 : 10 : 1); B. CHCl_3 : MeOH (94 : 6), then toluene : acetone (80 : 20); Stain: CuSO_4 (3%), H_3PO_4 (15%) in H_2O .

A. TLC of the EtOH extract of MAP and fractionations (Fr1~6).

B. Purification of Fr4 via reverse-phase HPLC.

C. HRMS data for Fr4-4~6.

Fr4-4 (Lipopeptide II α)

HRMS (TOF): calcd for C₅₁H₈₂N₅O₇ (M+H⁺): 876.6214, found: 876.6221

Fr4-5 (Lipopeptide II β)

HRMS (TOF) calcd for C₅₃H₈₆N₅O₇ (M+H⁺): 904.6527, found: 904.6535

Fr4-6 (Lipopeptide II γ)

HRMS (TOF) calcd for C₅₅H₉₀N₅O₇ (M+H⁺): 932.6840, found: 932.6849

Figure 2.

Isolation of new lipopeptides that exhibit serum antibody response activity.

A. Derivatization of amino acids with (S)-5.

B. HPLC separation of 6a-h.

C. LC-MS data for 6a-h.

substrate (4a-h)	HPLC peak number	elution order	retention time (T _R , min.)	ΔT _R (L-D) (min.)	LC-MS
[(S)-5]-L-Ala-OH (6a)	1	L>D	18.47	-0.38	466.5
[(S)-5]-D-Ala-OH (6b)	2	L>D	18.85		466.4
[(S)-5]-D-Ile-OH (6c)	3	D>L	22.05	+0.07	508.1
[(S)-5]-L-Ile-OH (6d)	4	D>L	22.12		509.0
[(S)-5]-L-Phe-OH (6e)	5	L>D	23.33	-0.71	542.4
[(S)-5]-D-Phe-OH (6f)	6	L>D	24.04		542.5
[(S)-5]-L-N-Me-Val-OH (6g)	7	L>D	25.28	-0.34	508.1
[(S)-5]-D-N-Me-Val-OH (6h)	8	L>D	25.62		508.1

Figure 3.

Analyses of the diastereomers of α-amino acids via HPLC.

Figure 4.
NOESY data for lipopeptide I (**1**).

Figure 5.
Conformational analyses of lipopeptide II β ; one of the most plausible conformers is shown.

Figure 6.
Serum antibody binding activity of lipopeptide II β probes.

Scheme 1.

Structures of lipopeptide II α , β , and γ , and methylation and amidation of lipopeptide II β (3).

Scheme 2.

Syntheses of 2α-aminolipopeptide IIβ and its fluorescent derivatives.