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Molecular Networking-Guided Discovery of Kyonggic Acids in *Massilia* spp.

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In recent years, bacteria of the genus *Massilia* have been identified as promising sources of bioactive natural products. In this study, we used mass spectrometry (MS)-based molecular networking for the first comprehensive analysis of the secondary metabolome of these microorganisms. Annotation of the detected metabolite clusters with a mass fragmentation database revealed several compounds not previously known to be produced by *Massilia* species. To secure sufficient material for further structural analyses as well as biological testing, we selected the most promising producer strains from our molecular networking analysis and subjected them to larger-scale cultivation. Following extraction and UV-directed isolation, we obtained five metabolites in milligram quantities. In addition to a cyclic dipeptide, we elucidated the structures of four acyl amino acids that are related to thalassotalic acid C from the marine bacterium *Thalassotalea* sp. The *Massilia*-derived kyonggic acids were found to inhibit the enzyme tyrosinase, which is involved in melanin biosynthesis influencing skin and hair pigmentation. This study illustrates the value of combining spectroscopic and computational tools in natural product discovery in order to detect and guide the isolation of previously overlooked bioactive compounds.

Introduction

The chemistry of microbial natural products is remarkably diverse and continues to provide valuable lead structures for drug development, as exemplified by the recent discovery of the antibiotic clovibactin.[1] Natural product-based drug discovery has been demonstrated to greatly benefit from the implementation of innovative approaches, such as the prioritization of underexplored taxa, or the integrated use of bioinformatics and metabolomics.[2] In the present study, we set out to systematically analyze the production of secondary metabolites by bacteria of the genus *Massilia*. This species-rich genus, whose members can be isolated from a variety of soil and freshwater environments, represents a largely untapped natural product resource. Although its biosynthetic potential was recognized in previous genomic analyses,[3] only three *Massilia* strains have actually been explored with regard to the production of secondary metabolites. These investigations led to the discovery of cyclic peptides, namely massiliamide[4] and the empedopeptins,[5] as well as alkaloids, including the structurally unusual massinidine[6] and the bisindole violacein.[7] The latter two compounds were isolated from a biosynthetically particularly talented strain, *Massilia* sp. NR 4-1, which also yielded the thiazoline siderophore massiliachelin.[8,9]

To obtain a broader view of the metabolic diversity within the genus, we collected metabolomics data from six *Massilia* strains and processed this data with the Global Natural Products Social Molecular Networking (GNPS) platform.[10] GNPS utilizes computational algorithms to construct spectral networks, wherein related compounds are grouped based on shared fragmentation patterns observed in tandem mass spectrometry (MS/MS) data. This network-based approach enables the visualization of intricate connections between different metabolites, thereby aiding in the identification of compounds with shared structural features or biosynthetic building blocks. Most importantly, the clustering of related compounds eliminates the need for time-intensive raw MS data analysis. By combining the molecular network from GNPS with an extensive reference spectra database, it is further possible to narrow down potential candidates for unknown metabolites. Using this approach, we identified a previously overlooked family of metabolites that was not known to be produced by *Massilia* strains. Five metabolites (Figure 1) could be isolated in milligram quantities and their structures were validated by NMR spectroscopy and mass spectrometry. Bioactivity testing revealed that compounds **1**–**4**, referred to as kyonggic acids, inhibit the enzyme tyrosinase.

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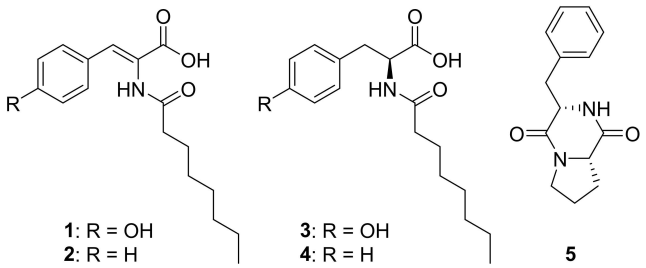

Figure 1. Chemical structures of compounds **1–5** isolated in this study.

Results and Discussion

For the metabolomic analysis, we chose six *Massilia* strains originating from different environmental habitats. Since the aim of this study was to cover at least parts of the diversity within the genus, the phylogenetic position of the strains was taken into account in the selection (Figure S1). The chosen strains were *Massilia flava* Y9, *M. kyonggiensis* TSA1, *M. namucuonensis* 333-1-041, *M. tieshanensis* TS3, *M. umbonata* LP01, and *Massilia* sp. NR 4-1. Except for the latter, none of the strains had been described as a producer of secondary metabolites before. The already investigated *Massilia* sp. NR 4-1 was included in this analysis due to the large number of biosynthetic gene clusters (BGCs) in its genome, for which no metabolic products had been reported.[8]

All chosen *Massilia* strains were grown in Reasoner's 2A (R2A) medium and the secreted metabolites were recovered by adsorption onto XAD-7 resin. The adsorbed molecules were eluted with methanol and the concentrated eluates were measured via LC-HRMS/MS. The resulting data of mass features were manually refined to exclude media components as well as trace compounds prior to the GNPS analysis.

Five mass features within the constructed molecular network matched entries in the GNPS library database (Figure S2), *i.e.*, they possessed the same molecular ion and the same fragmentation pattern. The library hits included *N*-acetyl-tryptamine, the cyclic dinucleotides cyclo(Tyr-Pro) and cyclo(Phe-Pro), as well as two *N*-acyl amino acids (NAAAs), namely *N*-tetradec-9-enoylglycine and *N*-pentadecanoyl phenylalanine (Figures S3–S7). While *N*-acetyltryptamine gave a single ion node in the molecular network, the other metabolites occurred in clusters of structurally related molecules. For instance, the GNPS library hit *N*-pentadecanoyl phenylalanine was found in a cluster comprising 25 nodes (Figure 2). Based upon their *m/z* values and fragmentation patterns the structures of seven further metabolites in this cluster were postulated. Since NAAAs had not been reported from *Massilia* spp. before, we set out to secure sufficient material of these compounds for structure elucidation and biological testing. Based upon the abundance of their mass features in the tested extracts (Figure 2), we concluded that *M. umbonata* LP01 and *M. kyonggiensis* TSA1 were the most promising strains for further analyses. We thus initiated fermentations in R2A medium at a 6-L scale. To stimulate NAAA biosynthesis in the two strains, their cultures were supplemented with L-tyrosine and L-phenylalanine. The extraction procedure was consistent with the protocol previously used in the metabolomics study. For further purification, the raw extracts were resuspended in water and extracted three times with ethyl acetate. The organic phases were pooled and concentrated under reduced pressure. The resulting residues were subjected to reversed-phase HPLC. The chromatograms showed that only *M. kyonggiensis* TSA1 had produced the target compounds in appreciable quantities (Figure S8). UV-guided chromatographic separation of the extract from this strain yielded four compounds (**1**–**4**).

Compound **1** was obtained as a brown amorphous powder. Its pseudomolecular ion at *m/z* 306.1716 $[M+H]^+$ and molecular formula of $C_{17}H_{23}NO_4$ were consistent with a mass feature that had already been detected in the molecular networking analysis (Figure 2). An inspection of the ^{13}C NMR spectrum revealed only 15 signals, but two of the resonances exhibited a comparatively high signal intensity. This suggested the presence of chemically equivalent carbon nuclei, as expected for a molecule featuring a *p*-substituted benzene moiety. The integrals and couplings of the aromatic proton signals supported this assumption. Overall, 1D and 2D NMR data confirmed the previously postulated structure, in which a dehydrotyrosine residue is *N*-acylated with caprylic acid (Figure 3). NOE correlations established the $\Delta^{2,3}$ double bond to be in *Z*-configuration. This finding is consistent with structurally related NAAAs, which were reported from other bacteria.[11–13]

Compound **2** was obtained as a white amorphous powder. It exhibits a $[M+H]^+$ ion at *m/z* 290.1766 corresponding to a molecular formula of $C_{17}H_{23}NO_3$. The ^1H and ^{13}C NMR spectra of **2** were closely resembling those of **1**, with the main differences pertaining to the aromatic region. A thorough inspection revealed that the *p*-substituted phenol moiety in **1** was replaced with a mono-substituted benzene in **2**. This was in full accordance with the observed mass loss of 16 Da in comparison to **1**. Therefore, it can be concluded that **2** is *N*-octanoyl dehydrophenylalanine.

Compound **3** was isolated as a white amorphous powder. It is structurally closely related to **1**, yet features two additional hydrogen atoms according to high-resolution (HR) ESIMS measurement. Its pseudomolecular ion at *m/z* 308.1870 $[M+H]^+$ is consistent with a molecular formula of $C_{17}H_{25}NO_4$. The absence of two sp^2-hybridized carbons and the appearance of two new aliphatic signals in the ^{13}C NMR spectrum strongly suggested that **3** lacks a $\Delta^{2,3}$ double bond and, hence, possesses a tyrosine residue. This assumption was confirmed after interpretation of the proton and 2D NMR spectra. To determine the configuration of **3**, its optical rotation was measured. The obtained value of $[\alpha_D^{20}] = +3°$ is consistent with the value reported for the structurally related *N*-myristoyl L-tyrosine ($[\alpha_D^{20}] = +2.1°$).[14] We thus propose that **3** is *N*-octanoyl L-tyrosine.

Compound **4**, which was obtained as brown amorphous powder, possesses a $[M+H]^+$ ion at *m/z* 292.1921 and a molecular formula of $C_{17}H_{25}NO_3$. Similar to **2**, the aromatic region in the ^1H NMR spectrum of **4** displays an AA′BB′C spin system, which is characteristic of a mono-substituted benzene. Further NMR analyses revealed that **4** is *N*-octanoyl phenyl-

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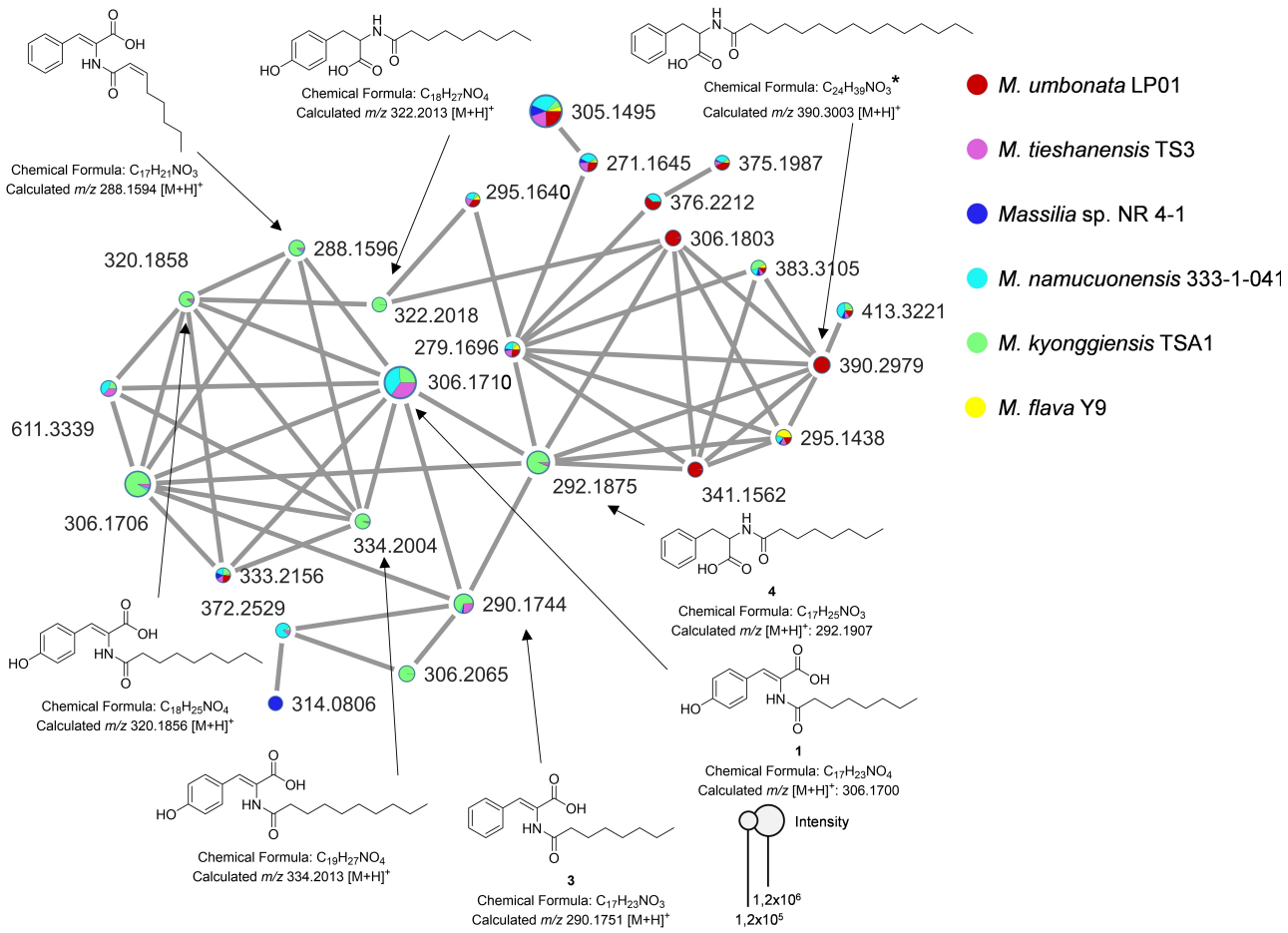

Figure 2. Feature-based molecular networking of *Massilia* culture extracts with the putative *N*-acyl amino acid (NAAA) cluster. Every circle represents a distinct mass feature. The circle size corresponds to the highest observed abundance of the respective mass feature in LC-MS/MS analysis. GNPS library hits are marked with an asterisk. The other depicted chemical structures were postulated based upon their *m/z* values and fragmentation patterns. The position and configuration of double bonds in the predicted structures are speculative.

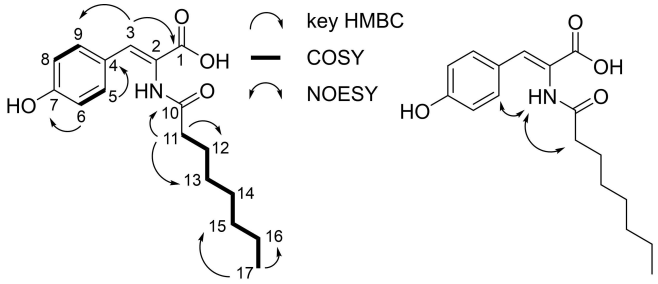

Figure 3. ^{1}H,^{1}H-COSY, as well as selected HMBC and NOESY correlations in **1**.

alanine. A measurement of the optical rotation $[\alpha_D^{20}] = +3°$ indicated that the amino acid has the L configuration.

The kyonggic acids **1**–**4** represent the first NAAAs isolated from a *Massilia* species. Compound **3** was described before from the yeast *Saccharomyces cerevisiae*[15] as well as from recombinant *Escherichia coli*[16] and *Pseudomonas* strains[17] expressing environmental DNA, while **4** was reported as an intermediate in the total synthesis of a syringolin A analog.[18] In contrast, compounds **1** and **2** represent new natural products according to a database search via SciFinder (https://scifinder-n.cas.org/). The dehydro-amino acid motifs, which occur in **1** and **2**, are rarely found in NAAAs. To our knowledge, the only examples described in the literature include the thalassotalic acids from the marine γ-proteobacterium *Thalassotalea* sp. PP2-459[11] and the stieleriacines from the marine planctomycete *Stieleria neptunia*.[13]

Our metabolic network analysis had already indicated that the potential for NAAA biosynthesis might not be restricted to *M. kyonggiensis* TSA1, the producer of **1**–**4**. An analysis of available *Massilia* genome sequences using antiSMASH[19] confirmed that NAAA biosynthesis genes are actually distributed in all strains, which must therefore be considered as potential NAAA producers. A complete list of NAAA biosynthetic gene clusters in *Massilia* genomes is provided in the Supplementary Information (Figure S10).

Up to now, the function of microbial NAAAs is not well understood. Some NAAAs were shown to act as antibacterial biosurfactants or to influence biofilm formation.[20] *N*-Acyl tyrosines and thalassotalic acids are also known to inhibit tyrosinase,[11,21] a key enzyme in melanin biosynthesis that influences skin and hair pigmentation.[22] Given the structural similarity of **1**–**4** to these inhibitors, their effect on tyrosinase

was tested. Arbutin, a natural product frequently found in skin-whitening products, served as a reference. While the IC_{50} values of **1** (176 ± 8 μM) and **3** (166 ± 9 μM) compared favorably to arbutin (131 ± 2 μM), **2** and **4** were much less potent tyrosinase inhibitors with IC_{50} values of 324 ± 5 and 355 ± 21 μM, respectively. From the results, we can conclude that the dehydrogenation is less important for the bioactivity in comparison to the hydroxylation of the aromatic ring.

Two further library hits, cyclo(Tyr-Pro) and cyclo(Phe-Pro) were found in a cluster with nine nodes (Figure 4). An inspection of the nodes next to the putative library hits suggested the presence of further cyclic dipeptides, namely cyclo(Leu-Pro), cyclo(Ile-Pro) and cyclo(Val-Pro). We confirmed the production of cyclo(Phe-Pro) (**5**) in a 6-L culture of *Massilia* sp. NR 4-1, which had been supplemented with L-tyrosine. The cyclic dipeptide was isolated as a beige amorphous powder. Its spectroscopic data (Table S2) were consistent with literature values.[23] The optical rotation of **5** ($[\alpha_D^{20}] = -51°$) was consistent with cyclo(L-Phe-L-Pro).[24]

Although proline-derived cyclic dipeptides have not been reported from *Massilia* bacteria until now, they are frequently found in microbial fermentations.[25] The biological function of these molecules remains unclear. In a filter disc assay against Gram-positive and Gram-negative bacteria, **5** showed no activity (Table S3). A search for cyclic dipeptide biosynthesis genes in *Massilia* genomes[26] did not return any hits. Since no proline-derived cyclic dipeptides were detected in our media control, we must assume that these compounds are due to biotransformation processes of media components occurring in all tested *Massilia* strains. Using the constructed metabolic network we then analyzed whether any previously reported *Massilia* natural product is commonly encountered in these bacteria. Massiliachelin[8] appeared to be the most likely candidate for a broad distribution, as siderophore pathways are often conserved in closely related organisms.[27] To our surprise, however, this was not the case. Mass features corresponding to massiliachelin,[8] massinidine,[8] and violacein[7] were exclusively found in culture extracts of *Massilia* sp. NR 4-1, whereas mass features corresponding to massiliamide[4] or empedopeptins[5] were completely missing. Although this result does not rule out a possible production under different fermentation conditions than those used in this study, it adds evidence to the assumption of a largely strain-specific secondary metabolome in the genus *Massilia*.

An interesting obervation made during these analyses concerned massiliachelin. In a previous study, it had been demonstrated that the production of this siderophore is accompanied by the formation of a number of biosynthetic shunt products.[9] Nevertheless, massiliachelin was found in a rather small cluster in the feature-based molecular networking, while the shunt products formed discrete clusters (Figure 5). The different clustering can be explained by distinct fragmentation patterns of these molecules. In addition to the discussed clusters, several strain-specific clusters were detected in the molecular network (Figure S2). The corresponding clusters did not include any GNPS library hit and may thus hold untapped potential for the discovery of new molecules.

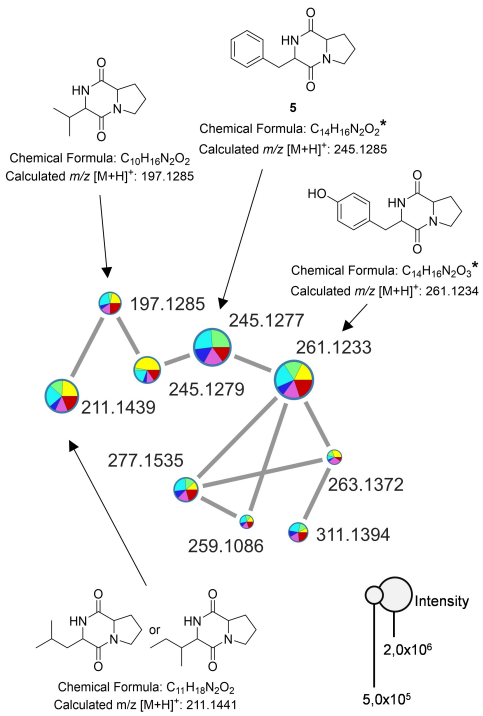

Figure 4. Feature-based molecular networking of *Massilia* culture extracts with the cyclic dipeptide cluster. Every circle represents a distinct mass feature. The circle size corresponds to the highest observed abundance of the respective mass feature in LC-MS/MS analysis. The color code links the detected mass features with the respective bacterial producer: blue, *Massilia* sp. NR 4-1; yellow, *M. flava* Y9; green, *M. kyonggiensis* TSA1; turquoise, *M. namucuonensis* 333-1-041; purple, *M. tieshanensis* TS3; red, *M. umbonata* LP01. GNPS library hits are marked with an asterisk. The other depicted chemical structures were postulated based upon their *m/z* values and fragmentation patterns.

Conclusions

In this study, we conducted the first comprehensive analysis of the secondary metabolome in *Massilia* spp. We compared the metabolic potential of six different *Massilia* strains using molecular networking. This network-based approach allowed us to explore intricate connections between various metabolites, facilitating the identification of compounds with similar structures or biosynthetic building blocks. Simultaneously, the mass features were compared with the vast reference spectra database, revealing several compounds not previously known to be produced by *Massilia* species. We confirmed the production of *N*-acylated amino acids, the kyonggic acids, aligning with bioinformatic analyses from *Massilia* spp. We also identified the cyclic dipeptide cyclo(L-Phe-L-Pro), which is likely due to a biotransformation of media components. Furthermore, we were able to identify compound clusters containing previously described metabolites, such as massiliachelin and its biosynthetic shunt products. In this project, we initially created a molecular network, followed by targeted analysis of the most promising bacterial strain. This strategy significantly enhanced our understanding of the biosynthetic capabilities of *Massilia* species.

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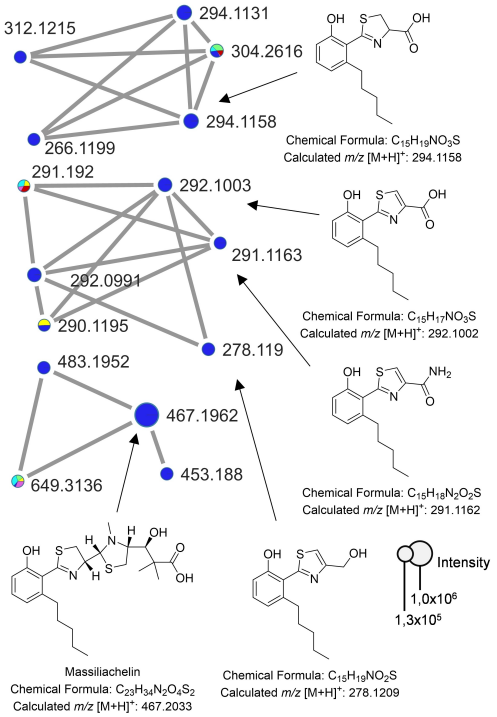

Figure 5. Feature-based molecular networking of *Massilia* culture extracts with clusters comprising massiliachelin and four of its biosynthetic shunt products. The MS/MS spectra of the depicted molecules were consistent with previously reported data.[8,9] Every circle represents a distinct mass feature. The circle size corresponds to the highest observed abundance of the respective mass feature in LC-MS/MS analysis. The color code links the detected mass features with the respective bacterial producer: blue, *Massilia* sp. NR 4-1; yellow, *M. flava* Y9; green, *M. kyonggiensis* TSA1; turquoise, *M. namucuonensis* 333-1-041; purple, *M. tieshanensis* TS3; red, *M. umbonata* LP01.

Experimental Section

General Experimental Procedures: Preparative HPLC was conducted on a Shimadzu LC-20A system equipped with two pumps (LC-20AD), a photo-diode array detector (SPD-M20A), a degasser unit (DGU-20A), an automatic sampler (SIL20A), and a column oven (CTO-20AC). LC-MS analysis was performed on a compact quadrupole-time of flight (Q-TOF) mass spectrometer from Bruker Daltonics with an Agilent 1260 Infinity LC system equipped with a Nucleoshell RP18 column (150×2.0 mm, 5 µm, Macherey-Nagel) NMR spectra was recorded on a Bruker 600 MHz Avance III HD system with DMSO-d_6 or CD_3OD as solvent and internal standard. The solvent signal was referenced to δ_H 2.50 ppm and δ_C 39.52 ppm for DMSO-d_6 and δ_H 3.31 ppm and δ_C 49.00 ppm for CD_3OD respectively. Optical rotation was measured at 20 °C on a PerkinElmer polarimeter 341 with a sodium lamp (wavelength = 589 nm) using a 1 dm cuvette. For this, samples were dissolved in methanol (1 mg/mL).

Bacterial Strains: *Massilia flava* Y9, *M. kyonggiensis* TSA1, *M. namucuonensis* 333–1-041, *M. tieshanensis* TS3, and *M. umbonata* LP01 were acquired from the Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures GmbH. *Massilia* sp. NR 4-1 was obtained from the Korean Collection for Type Cultures KCTC.

Cultivation and Extraction: The *Massilia* strains were cultivated in 250 mL Erlenmeyer flasks containing 50 mL of R2A medium: 0.5 g/L yeast extract, 0.5 g/L proteose peptone, 0.5 g/L casamino acids, 0.5 g/L glucose, 0.5 g/L soluble starch, 0.3 g/L KH_2PO_4, 0.05 g/L $MgSO_4$×7 H_2O. The pH of the medium was adjusted to pH 7.2. The cultivation was conducted on a rotary shaker at 180 rpm and 30 °C for five days. Afterwards, adsorber resin (XAD-7, 20 g/L) was added to the culture broth to bind the secreted metabolites. The resin was separated from the culture broth by filtration, washed with distilled water and exhaustively extracted with methanol.

Feature-Based Molecular Networking: The analysis of the mass features (MFs) was performed using Data Analysis 4.4 (Bruker, Bremen, Germany) and MZmine 3 R.[28] The processing steps for the raw data conversion and peak list generation in MZmine 3 is provided in Table S4 and Table S5. The *m/z* of the detected ions were grouped as pairs with the related retention time (rt) to generate a list of MFs (rt_*m/z*). For further analysis, MFs were only considered if they satisfied the following criteria: Intensity > 1000, available MS/MS-fragmentation and no detection in medium samples. From the generated MFs list, the exported MS/MS container file and feature quantification table from MZmine, a feature-based molecular network was created using the online workflow at Global Natural Products Social Molecular Networking (GNPS) in default mode.[10] For the molecular network, a precursor ion mass tolerance and fragment ion mass tolerance of 0.02 Da were used. Further network criteria were set as follows: minimum pairs cosine of 0.7; minimum matched fragment ions of 6; maximum shift between precursors of 500 Da and a network TopK of 10. The fragmentation spectra of the detected MFs in the network were compared against GNPS spectral libraries. Only matches with a score > 0.6 and at least 6 matched peaks were further considered. For the visualization of the molecular network, Cytoscape version 3.10.1 was used.[29] In the molecular network, Abundance ratios of each ion in the different samples were displayed as pie charts showing the highest observed intensity in each sample. The overall highest observed intensity of each ion in this study was presented additionally as circle size of the nodes. Molecular structures were generated with ChemDraw 20.0.

Production and Isolation of Metabolites: For metabolite production the strains were grown in 5 L Erlenmeyer flasks containing 1.0 L of R2A medium supplemented with 0.18 g/L L-tyrosine and 0.17 g/L L-arginine in the case of *Massilia* sp. NR 4-1 or 0.18 g/L L-tyrosine and 0.17 g/L L-phenylalanine in case of *M. kyonggiensis* TSA1 and *M. umbonata* LP01. The growth conditions and extraction followed the previously described procedure. The concentrated extracts from *Massilia* sp. NR 4-1 and *M. kyonggiensis* TSA1 were first fractionated by reversed-phase HPLC using a Nucleodur C18 Isis column (250×10 mm, 5 µm, Macherey-Nagel) and a gradient of acetonitrile in water supplemented with 0.1 % (v/v) trifluoroacetic acid. The gradient conditions were as follows: from 20 % acetonitrile to 90 % in 30 minutes and kept at 90 % for 10 minutes. The flow rate was set to 5 mL/min. The elution of compounds was monitored with a diode array detector over the range from 190 to 650 nm. If necessary, the relevant metabolites were further purified by isocratic fractionation with the same column, solvent, and flow rate, by lowering the concentration of acetonitrile by 10 % compared to the elution concentration that was achieved with the gradient method. For the purification of compound **4**, a Nucleodur Sphinx column (250×10 mm, 5 µm, Macherey-Nagel) with the above-mentioned gradient method was used. Compounds **1–4** were isolated from an *M. kyonggiensis* TSA1 culture extract. Compound **1** (2.8 mg) eluted after 13.9 min, followed by compounds **3** (1.7 mg, 15.7 min), **2** (1.6 mg, 18.8 min) and **4** (1.8 mg, 19.1 min) (Figure S8). Compound **5** (1.9 mg) was isolated from an *Massilia* sp. NR 4-1 culture extract and eluted after 6.4 min (Figure S9).

***N*-octanoyl (2*Z*)-dehydrotyrosine (1)**: ^{1}H NMR (600 MHz, DMSO-d_6, 300 K): δ = 7.47 (d, *J* = 8.8 Hz, 2H, CH-5 + CH-9), 7.18 (s, 1H, CH-3), 6.76 (d, *J* = 8.8 Hz, 2H, CH-6 + CH-8), 2.24 (t, *J* = 7.3 Hz, 2H, CH-11), 1.55 (qu, *J* = 7.3 Hz, 2H, CH-12), 1.30 (m, 2H, CH-13), 1.30 (m, 2H, CH-

14), 1.29 (m, 2H, CH-16), 1.27 (m, 2H, CH-15), 0.87 (t, $J=6.9$ Hz, 3H, CH-17); ^{13}C NMR (150 MHz, DMSO-d_6, 300 K): δ = 172.0 (C-10), 166.8 (C-1), 158.6 (C-7), 132.2 (C-3), 131.8 (C-5), 131.8 (C-9), 124.7 (C-4), 124.2 (C-2), 115.3 (C-6), 115.3 (C-8), 35.1 (C-11), 31.2 (C-15), 28.6 (C-13), 28.5 (C-14), 25.0 (C-12), 22.1 (C-16), 14.0 (C-17); HRMS (ESI): *m/z* calcd for $C_{17}H_{24}NO_4$ 306.1700 $[M+H]^+$; found: 306.1716.

***N*-octanoyl (2*Z*)-dehydrophenylalanine (2)**: ^{1}H NMR (600 MHz, DMSO-d_6, 300 K): δ = 7.60 (d, $J=7.1$ Hz, 2H, CH-5 + CH-9), 7.38 (t, $J=7.1$ Hz, 1H, CH-7), 7.35 (dd, $J=7.1$, 7.1 Hz, 2H, CH-6 + CH-8), 7.21 (s, 1H, CH-3), 2.25 (t, $J=7.3$ Hz, 2H, CH-11), 1.54 (qu, $J=7.3$ Hz, 2H, CH-12), 1.29 (m, 2H, CH-13), 1.29 (m, 2H, CH-14), 1.28 (m, 2H, CH-16), 1.26 (m, 2H, CH-15), 0.87 (t, $J=6.8$ Hz, 3H, CH-17); ^{13}C NMR (150 MHz, DMSO-d_6, 300 K): δ = 172.1 (C-10), 166.4 (C-1), 133.8 (C-4), 131.0 (C-3), 129.7 (C-5), 129.7 (C-9), 129.1 (C-7), 128.4 (C-6), 128.4 (C-8), 127.5 (C-2), 35.1 (C-11), 31.2 (C-15), 28.6 (C-13), 28.5 (C-14), 25.0 (C-12), 22.1 (C-16), 14.0 (C-17); HRMS (ESI): *m/z* calcd for $C_{17}H_{24}NO_3$ 290.1751 $[M+H]^+$; found: 290.1766.

***N*-octanoyl-L–tyrosine (3)**: ^{1}H NMR (600 MHz, DMSO-d_6, 300 K): δ = 6.98 (d, $J=8.3$ Hz, 2H, CH-5 + CH-9), 6.62 (d, $J=8.3$ Hz, 2H, CH-6 + CH-8), 4.28 (m, 1H, CH-2), 2.91 (dd, $J=4.9$, 14.0 Hz, 1H, CH-3a), 2.71 (dd, $J=9.6$, 14.0 Hz, 1H, CH-3b), 2.02 (t, $J=7.4$ Hz, 2H, CH-11), 1.39 (m, 2H, CH-12), 1.25 (m, 2H, CH-13), 1.25 (m, 2H, CH-14), 1.24 (m, 2H, CH-16), 1.19 (m, 2H, CH-15), 0.85 (t, $J=7.1$ Hz, 3H, CH-17); ^{13}C NMR (150 MHz, DMSO-d_6, 300 K): δ = 174.2 (C-1), 172.0 (C-10), 155.8 (C-7), 130.0 (C-5), 130.0 (C-9), 129.6 (C-4), 114.8 (C-6), 114.8 (C-8), 53.8 (C-2), 36.0 (C-3), 35.1 (C-11), 31.2 (C-15), 28.8 (C-13), 28.5 (C-14), 25.2 (C-12), 22.1 (C-16), 14.0 (C-17); HRMS (ESI): *m/z* calcd for $C_{17}H_{25}NO_4$ 308.1856 $[M+H]^+$; found: 308.1870.

***N*-octanoyl-L–phenylalanine (4)**: ^{1}H NMR (600 MHz, DMSO-d_6, 300 K): δ = 7.36 (t, $J=7.6$ Hz, 1H, CH-7), 7.24 (d, $J=7.3$ Hz, 2H, CH-5 + CH-9), 7.18 (dd, $J=7.3$, 7.6 Hz, 2H, CH-6 + CH-8), 4.37 (m, 1H, CH-2), 3.05 (dd, $J=4.8$, 13.8 Hz, 1H, CH-3a), 2.83 (dd, $J=9.6$, 13.8 Hz, 1H, CH-3b), 2.02 (t, $J=7.3$ Hz, 2H, CH-11), 1.37 (qu, $J=7.3$ Hz, 2H, CH-12), 1.25 (m, 2H, CH-13), 1.24 (m, 2H, CH-16), 1.17 (m, 2H, CH-15), 1.11 (m, 2H, CH-14), 0.86 (t, $J=7.0$ Hz, 3H, CH-17); ^{13}C NMR (150 MHz, DMSO-d_6, 300 K): δ = 173.2 (C-1), 172.0 (C-10), 138.0 (C-4), 129.1 (C-5), 129.1 (C-9), 128.0 (C-7), 126.2 (C-6), 126.2 (C-8), 53.4 (C-2), 36.8 (C-3), 35.1 (C-11), 31.1 (C-15), 28.4 (C-13), 28.4 (C-14), 25.2 (C-12), 22.0 (C-16), 14.0 (C-17); HRMS (ESI): *m/z* calcd for $C_{17}H_{25}NO_3$ 292.1918 $[M+H]^+$; found: 292.1921.

Cyclo-L–phenylalanyl-L–proline (5): ^{1}H NMR (600 MHz, DMSO-d_6, 300 K): δ = 7.31 (m, 2H, CH-10 + CH-14), 7.27 (m, 2H, CH-11 + CH-13), 7.25 (m, 1H, CH-12), 4.47 (ddd, $J=2.0$, 4.8, 4.8 Hz, 1H, CH-7), 4.09 (ddd, $J=2.0$, 6.4, 10.8 Hz, 1H, CH-2), 3.57 (dt, $J=8.4$, 11.8 Hz, 1H, CH-5b), 3.40 (ddd, $J=5.0$, 8.4, 11.8 Hz, 1H, CH-5a), 3.19 (dd, $J=4.8$, 4.8 Hz, 1H, CH-8b), 3.19 (dd, $J=4.8$, 4.8 Hz, 1H, CH-8a), 2.12 (m, 1H, CH-3b), 1.83 (m, 2H, CH-4), 1.25 (dtd, $J=8.7$, 10.8, 12.2 Hz, 1H, CH-3a); ^{13}C NMR (150 MHz, DMSO-d_6, 300 K): δ = 170.9 (C-1), 166.9 (C-6), 137.3 (C-9), 131.0 (C-11), 131.0 (C-13), 129.5 (C-10), 129.5 (C-14), 128.1 (C-12), 60.1 (C-2), 57.7 (C-7), 46.0 (C-5), 38.2 (C-8), 29.4 (C-3), 22.8 (C-4); HRMS (ESI): *m/z* calcd for $C_{14}H_{17}N_2O_2$ 245.1285 $[M+H]^+$; found: 245.1305.

Tyrosinase Inhibition Assay: The inhibitory effects of compounds **1–4** on mushroom tyrosinase were evaluated through the conversion of L-tyrosine to dopachrome, following a previously established method.[11] In a 96-well flat-bottomed microtiter plate, 40 μL of each test substance (serially diluted from 2 mM to 0.002 mM) dissolved in 10% methanol in phosphate buffer solution (PBS; 0.1 M, pH 6.8) was aliquoted. Subsequently, 80 μL of PBS, 40 μL of tyrosinase (100 units/mL, dissolved in PBS), and 40 μL of L-tyrosine (2.5 mM, dissolved in PBS) were added. Arbutin (Thermo Fisher Scientific Inc., United States of America) served as positive control. A blank, containing all components except L-tyrosine, accompanied each sample. The results were compared with a control that included 10% methanol instead of the test compounds. The mixture was gently shaken for 30 min at 37 °C, and absorbance was measured at 490 nm using a FLUOstar Omega from BMG Labtech. The values were plotted, and IC_{50} values were calculated. All tests were conducted in triplicate.

Antimicrobial Assay: The test bacteria, *Bacillus subtilis* DSM 168, *Escherichia coli* DSM 18039 and *Pseudomonas fluorescens* DSM 11532, were acquired from the Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures GmbH. To test antibacterial activity, 0.1 mL of an overnight culture of each bacterial strain was plated on agar plates containing their preferred medium. The samples being tested were dissolved in methanol at a concentration of 50 μg/10 μL. Each compound was impregnated onto filter papers at a volume of 10 μL per disc. The inoculated plates were then incubated at 30 °C for 48 hours. The antibacterial activity was evaluated by measuring the zone of inhibition against the test organism. Ampicillin (Roth, Carl Roth GmbH + Co. KG, Germany), tetracycline (Fluka Honeywell International Inc., United States of America) and ciprofloxacin (Sigma, Sigma-Aldrich Chemie GmbH, Germany) were used as positive controls.

Supporting Information

Spectroscopic data are provided in the Supporting Information. Additionally, the Supporting Information includes the entire molecular network, HPLC results, a phylogenetic analysis of *Massilia* spp., information on NAAA biosynthesis genes in *Massilia* genomes, the findings from the antimicrobial assay and the raw data processing parameters. Additional references cited within the Supporting Information.[30–33]

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: *N*-acyl amino acid · metabolome · molecular network · *Massilia* · structure elucidation

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