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N-Acyl derivatives of Asn, new bacterial *N*-acyl *D*-amino acids with surfactant activity

Short Communication

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Summary. New *N*-acyl *D*-amino acids were isolated from *Bacillus pumilus* IM 1801. Their structures were determined by chemical analysis and mass spectrometry. The lipid part was identified as a mixture of fatty acids with 11, 12, 13, 15, and 16 carbon atoms in the *iso*, *anteiso* or *n* configuration linked by an amide bond with a *D*-asparagine. They exhibited surfactant properties.

Keywords: *N*-acyl *D*-amino acids – Mass spectrometry – *Bacillus pumilus* – Surfactant activity

Introduction

N-acyl amino acids, also named as lipoamino acids, are widely distributed in microorganisms. Most of them contains a β-OH fatty acid linked via an amide bond to the α-amino group of the amino acid. A second fatty acid may esterify the hydroxy group of the first acid. This group includes *N*-acyl serine, glycine, lysine and ornithine (Asselineau, 1991). More recently, a new series of *N*-acyl leucine (or isoleucine) derivatives has been isolated from *Deleya marina* (Yagi et al., 1995). They distinguish from the first ones by their structures which do not contain a β-OH fatty acid and by their location in the cytoplasm of the cell instead of the cytoplasmic membrane (Yagi et al., 1997). The role of these compounds in the bacterial cell remains unknown. However, *N*-acyl ornithine, which is widely distributed in Gram-negative bacteria, has been proposed as a substitute of phosphatidylethanolamine under phosphate limitation (Yagi et al., 1997). In this context, it should be recalled that the *N*-acyl homoserine lactones are bacterial signalling molecules involved in regulating diverse metabolic functions (Robson et al., 1997). Because of their amphiphilic character, the lipoamino acids exhibit antibacterial, haemolytic and hemagglutining properties (Kawai et al., 1983, 1988; Kimura et al., 1969). Numerous *N*-acyl amino acids have been synthesized and tested for their surfactant properties and their interactions with artificial membranes (Miyagishi et al., 1989; Sanson et al., 1987; Epand et al., 1998). In mammals, *N*-acyl amino acids containing arachidonic acid have been recently demonstrated to suppress the inflammatory pain (Huang et al., 2001) and a potent adjuvant activity has been discovered for *N*-acyl ornithine (Kawai et al., 1999). *N*-acyl amino acids then display an array of various and amazing activities. In the course of our screening of new microbial surfactants from *Bacillus sp*., we have found that *Bacillus pumilus* IM 1801 produced new *N*-acyl amino acids. Moreover, they distinguished from the formers by their extracellular production.

This paper describes the production, the isolation and the structure elucidation of these new compounds using mass spectrometry. Due to the very low yield of production, we chemically synthesized homologues and we include data on the individual surface properties of these molecules.

Materials and methods

Culture conditions and extraction of the lipoamino acids

Bacillus pumilus IM 1801 was obtained from Institut Mérieux, Lyon, France. The slant culture of *Bacillus pumilus* IM 1801 was inoculated into Erlenmeyer flasks containing 50 mL of brain/heart infusion

(bioMérieux). The flasks were cultivated on a rotary shaker at 350 rpm for 24 h. The seed culture (30 mL) was transferred into a grooved Erlenmeyer flask containing 500 mL of the production medium containing per L: glucose 20 g, *L*-glutamic acid 5 g, $MgSO_4$ 0.5 g, KH_2PO_4 1 g, $Fe_2(SO_4)_3$ 1.2 mg, $MnSO_4$ 0.4 mg, $CuSO_4$ 1.6 mg. The pH is adjusted at 7.5 with NaOH 3M before sterilization. After 48 h of growth at 32°C, the culture broth was centrifugated. On one hand, the supernatant was adjusted to pH 2.0 with 6M HCl. The pellets collected after centrifugation were neutralized and lyophilised. On the other hand, the bacteria were also lyophilised. Both lyophilisates were extracted twice with chloroform/methanol (2:1). Crude extracts from both the bacteria and the culture filtrate were analysed by TLC on silica gel 60 (Merck) in chloroform-methanol-H_2O (65:25:4, by vol.). *N*-acyl amino acids were detected as two white spots, after spraying water and medium heating. Each spot was then subjected to chemical analysis and MS for structure determination.

Structure elucidation

The *N*-acyl amino acids were hydrolyzed by 6M HCl at 110°C for 18 h. The hydrolyzates were extracted with chloroform and both a lipid and a water-soluble parts were obtained. The analysis of the water-soluble part of each spot was carried out by TLC on cellulose powder (Merck) in isopropanol/pyridine/acetic acid/water (40:40:5:20, by vol.).

The chirality of aspartic acid was assessed out by a precolumn derivatization procedure using *ortho*-phtalaldehyde (OPTA) and *N*-acetyl-*L*-cysteine (AcCys) as reagents (Nimura et al., 1986). Analysis was carried out on a Kromasil 5 C_{18} column (150 × 4.6 mm). Elution was performed by mixing 50 mM sodium acetate pH 5.9 and MeOH in proportions controlled by a microprocessor gradient program at a flow rate of 1 mL/mn and, the detection was made at 332 nm.

The lipid moieties were derivatized to methyl esters with gaseous diazomethane and the fatty acid methyl esters were analysed by GC/MS (BP 5 capillary column (25 m × 0.22 mm) with helium as carrier gas and temperature programming from 120°C (5 min) to 280°C at 4°C/min.

Mass spectrometry

EI-MS of fatty acid methyl esters was performed at 200°C and 70 eV with an AEI MS 50 instrument.

FAB mass spectra of the lipoamino acids were obtained with a Kratos MS 80 mass spectrometer. The ion source was equipped with an Ion Tech atom gun. Bombardment was realized with xenon atoms at 6–7 kV energy. Samples were introduced on a copper probe tip using a glycerol/*meta*-nitrobenzyl alcohol (50:50) mixture (1 μl). When necessary, a small amount of NaCl or LiI solutions was added to the mixture, prior to the analysis.

MS/MS spectra were recorded using a ZabSpec-T five-sector tandem mass spectrometer (Micromass, Manchester, UK) with $E_1B_1E_2$-B_2E_3 geometry (E: electrostatic analysers, B: magnets). Samples were dissolved in the same matrix as for the FAB-MS experiments. $[M+H]^+$, $[M+Na]^+$ or $[M+Li]^+$ precursors ions were generated by cesium ion bombardment at 30 KeV. The precursor ion submitted to MS/MS experiments were selected by MS1 set at appropriate E and B values and then focused in a collision cell located in the fourth field-free region (between E_2 and B_2). Argon was introduced at a pressure leading to an almost 70% attenuation of the precursor ion beam. The collision cell was floated at 4 kilovolts in order to get a collision energy of 4 KeV.

Chemical synthesis

Briefly, *N*-acyl amino acids were prepared by adding acyl chloride (lauroyl or palmitoyl) dropwise to an aqueous solution of the sodium salt of *D*-asparagine at pH 10, previously cooled at 0°C, under moderate stirring (Molinero et al., 1988). After addition, the mixture was then stirred for 5 hours at room temperature. *N*-acyl *D*-asparagines were purified by column chromatography on silica gel with chloroform/methanol mixtures as solvents.

Surface tension measurement

Assays were performed with the synthetic *N*-acyl amino acids, dissolved in 5 mM Tris pH 9,5 (Grangemard et al., 1999). Surface tensions were measured with a Krüss tensiometer using the procedure of Du Nouy with a platinum ring at 25°C. The surface tension-concentrations plots were used to determine the critical micellar concentration (CMC) and the surface tension close to CMC (γ_{CMC}).

Results and discussion

Analysis of the crude extracts, either from the bacteria or the culture broth, by TLC on silica gel in the solvent chloroform-methanol-H_2O (65:25:4, by vol.) showed the presence of two compounds (Rf = 0.35 and 0.40), detected by spraying water or dimethylaminobenzaldehyde reagent. Moreover, negative reaction to phosphomolybdate reagent clearly distinguished them from the phospholipids present in the bacterial extract. The spots were scrapped off the gel and eluted with methanol. By preparative TLC in the same solvent, about 4 mg of product were obtained from 1 L of the culture broth and 1 mg from the bacteria.

The gas chromatograms of the lipid parts are shown in Fig 1. They resolved into six fatty acids for the spot 1, Rf = 0.35 (Fig. 1A) and five fatty acids for the spot 2, Rf = 0.40 (Fig. 1B). They were identified by their retention times and molecular masses, by comparison with standards. From the spot 1, minor compounds 1 (3%), 2 (5%), 3 (4%) were identified as *iso*, *anteiso* and *n* C_{11}, the compound 4 (5%) was *n* C_{12} while the compounds 5 (66%) and 6 (17%) corresponded to *iso* C_{13} and *n* C_{13}, respectively. The spot 2 chromatogram revealed a major compound 11 (61%) identified as *n* C_{16}, together with compound 10 (6%) corresponding to *iso* C_{16} while compounds 7 (12%), 8 (13%), 9 (8%) were *iso*, *anteiso*, *n* C_{15}, respectively. It should be noted that the relative proportions of the fatty acids exhibited some variability according to the culture. Especially, the C_{15} chain was sometimes absent. However, in any case, the *iso* C_{13} and *n* C_{16} remained the major lipids.

Concerning the hydrolysates of the spots 1 and 2, aspartic acid, issuing from asparagine, was identified as the unique amino acid. Consequently, the difference between the Rf values of both spots, observed by TLC, was not due to a difference in the structure of the water-soluble part but to a difference in the structure of the lipidic part, i.e. aliphatic chains with different lengths.

The chirality of aspartic acid was determined by reverse-phase HPLC of OPTA-AcCys derivatives. Figure 2 shows the HPLC profiles of the hydrolyzate alone and in mixture with each of the standard enantiomer, *D*- and *L*-aspartic acid. They clearly evidenced the *D* chirality of the amino acid constituting the hydrophilic part of the molecule.

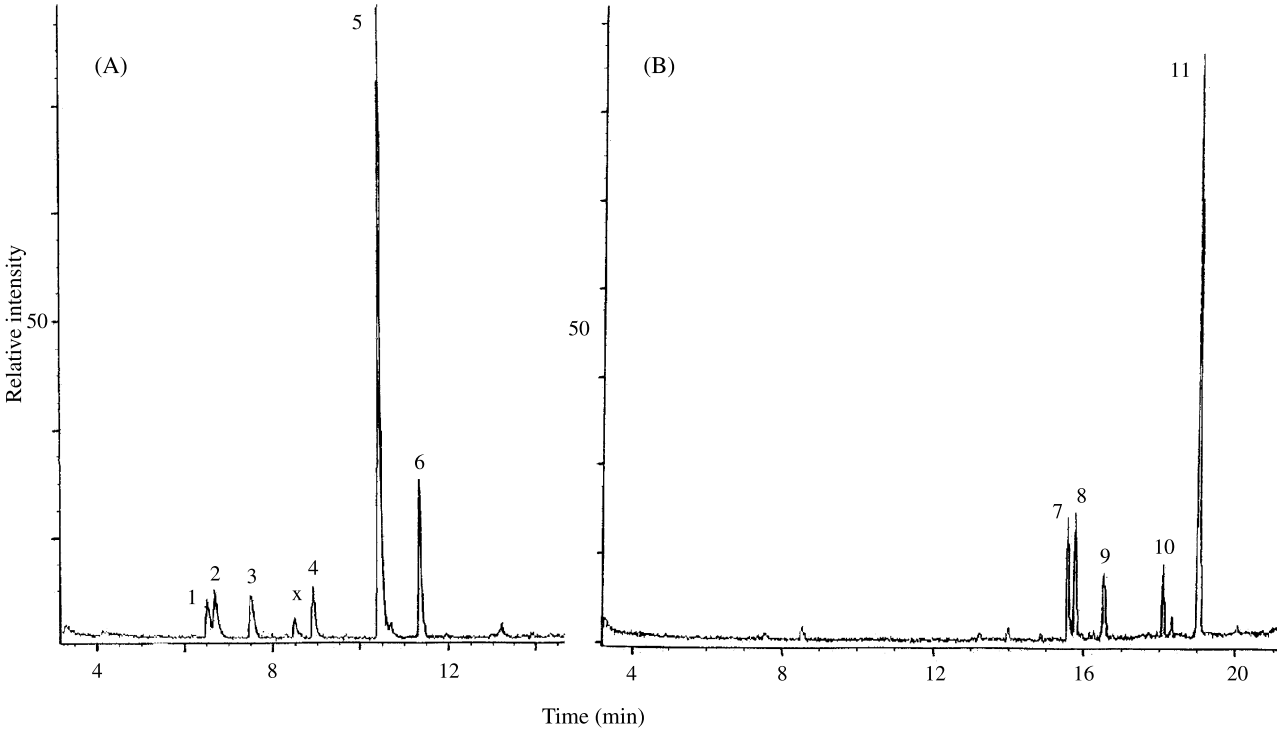


Fig. 1. Gas chromatograms of the methyl esters of fatty acids constituting the lipid parts, (**A**) from the spot 1 and (**B**) from the spot 2. (*1*) *iso*C_{11}; (*2*) *anteiso*C_{11}; (*3*) *n*C_{11}; (*4*) *n*C_{12}; (*5*) *iso*C_{13}; (*6*) *n*C_{13}; (*7*) *iso*C_{15}; (*8*) *anteiso*C_{15}; (*9*) *n*C_{15}; (*10*) *iso*C_{16}; (*11*) *n*C_{16}, (*X*: unidentified)

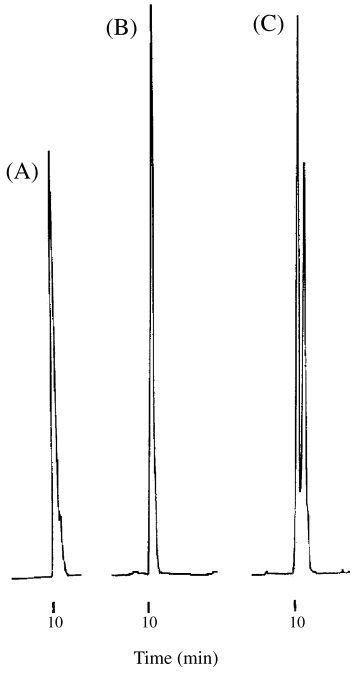


Fig. 2. HPLC profiles of OPTA-AcCys derivatives of (**A**) hydrolyzate alone; (**B**) hydrolyzate in mixture with standard *D*-Asp; (**C**) hydrolyzate in mixture with standard *L*-Asp

The structure of the *N*-acyl amino acids was determined by mass spectrometry and by comparison of the molecular masses and the Rf values with those of synthetic *N*-lauroyl or *N*-palmitoyl *D*-asparagines.

Mass spectrometry was performed on each spot obtained by TLC. In some cases, addition of NaCl or LiI to the matrix allowed the obtention of the cationized species.

Firstly, it was observed that all the spectra displayed odd molecular masses. Based on the presence of aspartic acid in the hydrophilic moiety, it was therefore suspected the presence of the amide form of the amino acid. Indeed, in mass spectrometry, the rule concerning nitrogen is that the molecular weight will always be even if the number of nitrogen atoms is zero or even. Any addition of a nitrogen atom in a molecule will shift the parity, which is the case when a carboxyl group is amidated. Secondly, as expected from the GC analysis, the spectra exhibited a pattern with mass shifts of 14 Da, due to the mixture of the molecules varying in the lipidic moiety.

FAB mas spectrum of the compounds extracted from the spot 1 showed, by addition of sodium chloride, a series of $[M + Na]^+$ ions at $m/z = 323$, 337, 351, and their

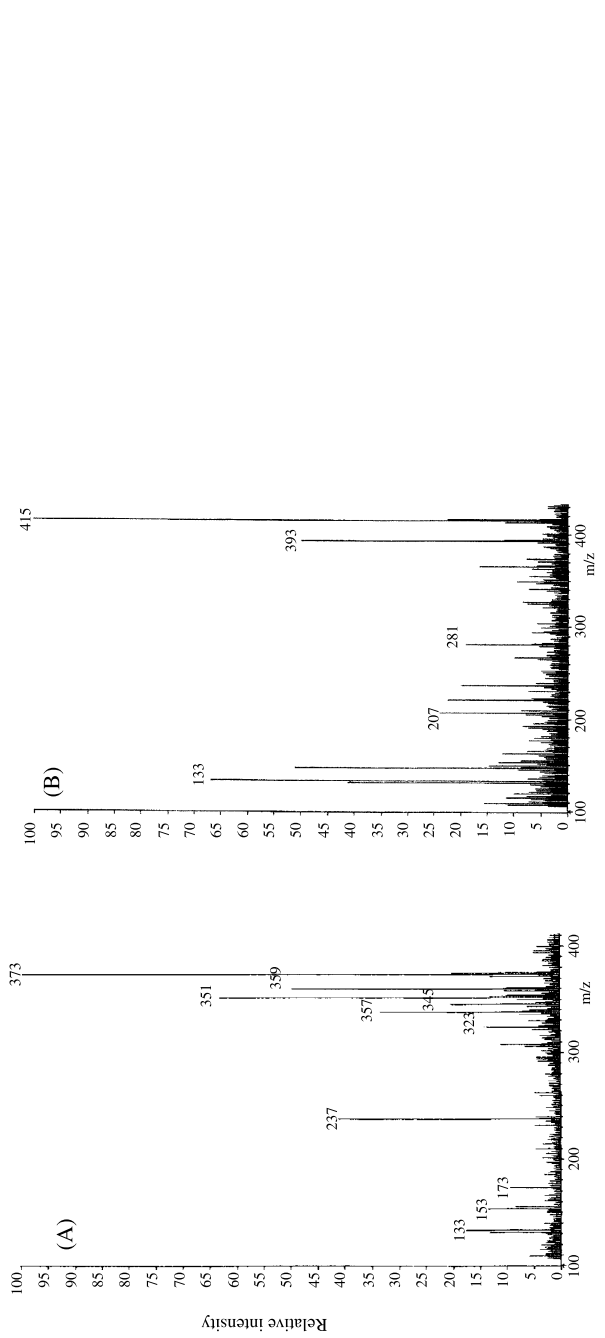
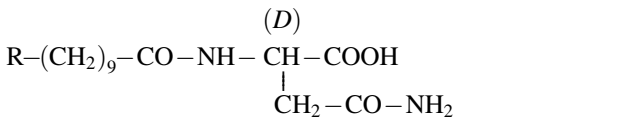


Fig. 3. FAB mass spectra of the lipoamino acids. (**A**) and (**B**): mass spectra of the cationized species: $[M + Na]^+$ at $m/z = 323$ (C_{11}), 337 (C_{12}), 351 (C_{13}), 393 (C_{16}) and $[M + 2Na - H]^+$ at $m/z = 345$ (C_{11}), 359 (C_{12}), 373 (C_{13}) and 415 (C_{16}); (**C**) mass spectrum of the protonated species (spot 2) at $m/z = 371$; (**D**): MS/MS spectrum obtained from the protonated ion precursor at $m/z = 371$

corresponding $[M+2Na-H]^+$ ions at $m/z=345$, 359, 373. These values were in agreement with an acyl chain of 11, 12 and 13 carbon atoms, respectively, linked by an amide bond to asparagine (Fig. 3A).

From the spot 2, the cationized ions at $m/z=393$ ($[M+Na]^+$) and $m/z=415$ ($[M+2Na-H]^+$) indicated the presence of an acyl chain with 16 carbon atoms (Fig. 3B). To support the presence of asparagine in the structure, the MS/MS spectrum was recorded on the latter compound in its protonated form ($m/z=371$, $[M+H]^+$) (Fig. 3C). Collision-induced dissociation of this precursor ion led to an intense fragment ion peak at $m/z=133$ which was attributed to the protonated asparagine [H–HN–CH–(CH_2–CO–NH_2)–COOH + H^+] obtained by the fragmentation of the amide bond (Fig. 3D). This result was confirmed by the MS/MS spectrum of the ion precursor $[M+Li]^+$ ($m/z=377$). A peak at $m/z=139$, i.e. 6 mass units higher than the above-mentionned one ($m/z=133$) was evidenced (spectra not shown).

From all these results, the basic structure was proposed:



with R = $CH_3-\underset{\underset{CH_3}{|}}{CH}-$ (*iso* C_{13}), $CH_3-(CH_2)_5-$ (*n*C_{16})

and other minor chains.

Concerning the properties of these compounds, various activities were tested. No antibacterial or antifungal activities were found. Conversely, they exhibited surfactant property with a γ_{CMC} at about 42 mN/m at 25°C. Due to the very low production of these products by the bacterium, we synthetized individual homologues. As an example, the results obtained with the anionic form (5 mM Tris pH 9.5) of the lauroyl and palmitoyl *D*-asparagines are shown in Fig. 4. The CMC values are very close, i.e. 2.9 and 2.7 mM, respectively, while the γ_{CMC} varies from 46.5 mN/m for the C_{12} chain to 41 mN/m for the C_{16} chain, corresponding to a decrease of about 1 mN/m per methylene group. Furthermore, two other breaks occuring at lower concentrations suggest premicellization phenomenon. The small difference between the CMC values is somewhat surprising. Indeed, if we consider the literature data, it has been observed that the CMC values generally decrease by a factor 10 when alkyl chain is increased by two carbon atoms (Tanford, 1980). This apparent discrepancy may be due to low solubility and to the absence of any added salt.

The role and the metabolism of these compounds remain unknown. Recently, a metalloenzyme from *Pseudomonas* sp. was found to catalyze the hydrolysis of *N*-acyl *D*-glutamate (Wakayama et al., 1995a, b). This enzyme is industrially used for the production of *D*-Glu. Several *N*-acyl *D*-aminohydrolases have been found in other bacterial species including *Alcaligenes*, *Streptomyces*, *Stenotrophomonas* and the corresponding encoding genes were cloned and sequenced (Muniz-Lozano et al., 1998; Lin et al., 2002;). An interest of these compounds is their potential use in food industry. Indeed, together with their bactericidal or surfactant properties (Takehara et al., 1972), some *N*-acyl *D*-amino acids can serve as adducts or substitutes to sodium nitrite in meat processing (Paquet et al., 1987). In this context, it should be recalled that the *N*-acyl sarcosinate surfactants are commercially available and used for many years in care and cleaning products (King, 1954; Cramer and Smith, 1987).

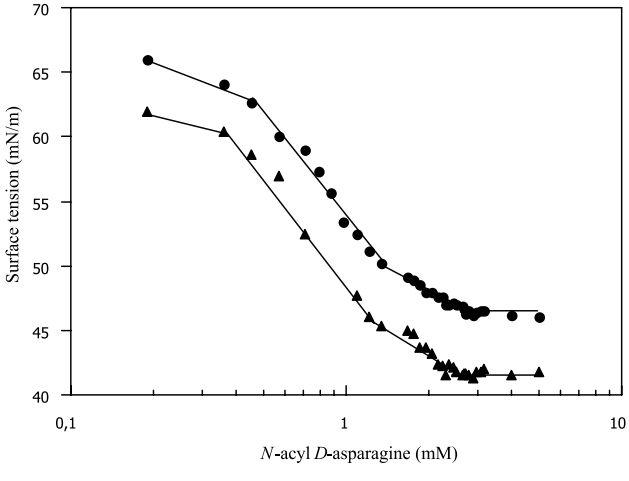


Fig. 4. Surface tension *versus* concentrations of *N*-lauroyl *D*-Asparagine (●) and *N*-palmitoyl *D*-asparagine (▲)

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