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Hemolytic activity of new linear surfactin analogs in relation to their physico-chemical properties

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Abstract

New linear analogs of surfactin have been synthesized. Their physico-chemical parameters were determined. The results indicate that these linear products show surface activities although they are lowered compared to those of cyclic compounds. The hemolytic activities have also been assayed. In contrast with cyclic surfactins, no significant hemolysis occurs for the linear products in the range of concentrations tested. Moreover, a protective effect against Triton X-100 induced hemolysis has been highlighted for linear surfactins. The concentration at which this protective effect happens is correlated directly to the CMC, and inversely to the acyl chain length of the product. In a hypotonic medium, analogs having a long acyl chain tend to increase the hemolysis, meanwhile the product with the shortest chain tends to decrease it.

Keywords: Surfactin; CMC; Hemolysis; Surfactin analog

1. Introduction

Surfactin is a lipopeptide family excreted by *Bacillus subtilis*. Its structure is characterized by a heptapeptidic moiety linked to a beta hydroxyl-fatty acid. A lactone bridge between the beta-hydroxyl function of the acid and the carboxy-terminus function of the peptide confers a cyclic structure to this molecule [1–4] (see Fig. 1a). A natural diversity occurs, giving rise to homologs, differing from each other by the length (13 to 15 atoms of carbon) and the ramification of the fatty acid chain; and to isoforms, characterized by some differences in the peptidic sequence.

The increasing interest for these molecules is due to their amphiphilic character, which is responsible for their excellent surface-active properties [5–7]. In addition, surfactins exhibit diverse biological activities such as antiviral [8–11], antibacterial [12], antimycoplasma [8,9,13] and hemolytic activities [10,14,15]. However, this last property constitutes a drawback for medical applications.

The aim of this work was to use the possibilities offered by organic synthesis to vary the structure of surfactins in order to lower their hemolytic effect. Linear surfactins have been chosen as the first series of analogs. These molecules can be compared with a natural linear surfactin obtained by hydrolysis of the lactone function of surfactin. Some authors have already studied the surface-active properties [16,17]

Abbreviations: CHCA, α-cyano 4-hydroxy cinnamic acid; CMC, critical micellar concentration; DCM, dichloromethane; DIEA, di-isopropyl ethylamine; DMSO, dimethyl sulfoxide; HC_{50}, concentration inducing 50% of hemolysis; HOBt, n-hydroxybenzotriazole; NMP, N-methyl-pyrrolidone; PC_{50}, concentration inducing 50% of protection; RBC, red blood cells; SAL14, synthetic linear surfactin with an acyl chain of 14 atoms of carbon in which the carboxy terminus function has been amidated; SNC14, natural cyclic surfactin with an acyl chain of 14 atoms of carbon; SNC15, natural cyclic surfactin with an acyl chain of 15 atoms of carbon; SNL14, linear surfactin obtained by hydrolysis of SNC14; SSL10, synthetic linear surfactin with an acyl chain of 10 atoms of carbon; SSL14, synthetic linear surfactin with an acyl chain of 14 atoms of carbon; SSL18, synthetic linear surfactin with an acyl chain of 18 atoms of carbon; TBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; TX-100, Triton X-100

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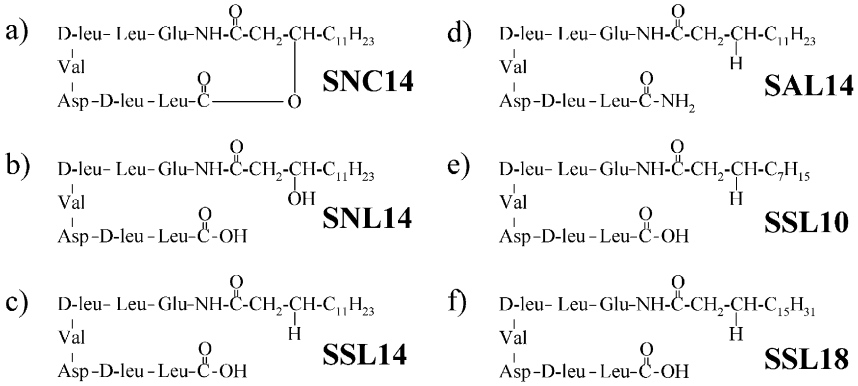

Fig. 1. Structure of the different molecules studied. (a) SNC14 or cyclic natural surfactin with a C14 acyl chain. (b) SNL14 or chemically linearised natural surfactin with a C14 acyl chain. (c) SSL14 or linear synthetic surfactin with a C14 acyl chain. (d) SAL14 or linear synthetic surfactin with an amidated carboxy-terminus function and a C14 acyl chain. (e) SSL10 or linear synthetic surfactin with a C10 acyl chain. (f) SSL18 or linear synthetic surfactin with a C18 acyl chain.

and the secondary structure [17] of a linear surfactin obtained by hydrolysis of natural cyclic surfactin. The linear surfactins used in this work are of synthetic or natural origins, have a unique and well-defined structure and differ from each other by the length of the acyl chain, or the number of ionisable acid functions.

2. Materials and methods

2.1. Chemicals

Each of these products was purchased from the following companies: fmoc-leucyl wang resin and fmoc protected amino acid, from Advanced Chemtech, USA; HOBt, 98%, from Eurobiochem, Germany; TBTU, 98%, from Alexis, Switzerland; TFA, synthesis quality, from SDS, France; Tris, 99.8%, from Pharmacia Biotech, Sweden; CHCA, from Sigma, USA; TX-100, from Sigma, USA; milliQ water, obtained with a millipore synthesis A10 apparatus, from Millipore, USA. Capric anhydride, 98%; DIEA, 98%; stearic anhydride, 98%; and hydrochloric acid, 37% in water, were purchased from Acros Organics, USA. DMSO, 99.9%; myristic anhydride, 95%; and piperidine, 99%, were purchased from Aldrich, Germany. Acetonitrile, HPLC quality; DCM, multisolvant; methanol, multisolvant; NaCl, reagent grade; and NMP, for peptide synthesis, were purchased from Sharlau, Spain.

2.2. Production of surfactin analogs

Surfactin was extracted from a *Bacillus subtilis* S499 culture supernatant and purified by chromatography as previously described [18,19].

Natural linear surfactin with a fatty acid chain having 14 carbon atoms (SNL14, see Fig. 1 for the structure) was obtained by alkaline hydrolysis of pure natural cyclic surfactin having the same C14 fatty acid chain (SNC14) in methanol: 0.1 M NaOH 4:6 (v/v) at 37 °C during 18h, according to the procedure described by Morikawa et al. [16].

The production of the different synthetic linear analogs has been performed by the classical SPPS technique. An advanced chemtech 348 MPS (Advanced Chemtech, USA) apparatus was used. A wang resin, supporting 50 μmol of fmoc-protected leucine, was treated with piperidine 20% in NMP to remove the fmoc protecting group. Attachment of the next amino acid was performed by adding 1.5 ml NMP/DMSO/DIEA 82:16:2 (v/v/v) containing 150 μmol of the corresponding fmoc-protected amino acid, 150 μmol of HOBt and 145 μmol of TBTU. This coupling reaction was repeated twice. The procedure was the same for the attachment of each amino acid. At the end of its synthesis, the peptidic moiety was treated with 75 μmol of, capric-, myristic- or stearic-anhydride, respectively, in DCM, in order to obtain synthetic linear surfactins with C10, C14 and C18 chain length (SSL10, SSL14 and SSL18). This reaction was repeated fivefold. The lipopeptide was cleaved from the resin with TFA/DCM/water 49.5:49.5:1 (v/v/v). After filtration, the liquid phase was evaporated under nitrogen and dissolved in methanol. The lipopeptide was precipitated by dropwise addition of water.

Linear synthetic surfactin in which the carboxy-terminus moiety has been amidated (SAL14) was obtained using the same procedure on a rink amide resin which gives rise to amidated peptide once cleaved with TFA.

The purification of each product was performed by preparative HPLC (prep LC 4000, Waters, USA), using a 22×250 mm C18 or C4 column (Vydac, USA) with various gradients of acetonitrile/milliQ water 0.05% TFA. The obtained fractions were evaporated under vacuum, diluted with milliQ water and freeze dried. Identification and purity of the products were attested using infrared spectroscopy, amino acid analysis and rp-HPLC as described previously [18]. Mass spectra were taken from a solid spot containing the lipopeptide in a CHCA matrix with an MALDI-TOF mass spectrometer (ultraflex TOF, Bruker, Germany).