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Synthesis and Characterization of a Lipidic Alpha Amino Acid: Solubility and Interaction with Serum Albumin and Lipid Bilayers

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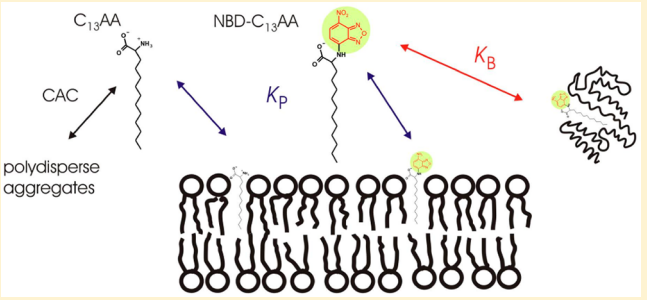
Supporting Information

ABSTRACT: The lipidic α-amino acid with 11 carbons in the alkyl lateral chain (α-aminotridecanoic acid) was synthesized via multicomponent hydroformylation/Strecker reaction, which is a greener synthetic approach to promote this transformation relative to previously described methods. Its solubility and aggregation behavior in aqueous solutions was characterized, as well as the interaction with lipid bilayers. Lipidic amino acids are very promising molecules in the development of prodrugs with increased bioavailability due to the presence of the two polar functional groups and nonpolar alkyl chain. They are also biocompatible surfactants that may be used in the food and pharmaceutical industry. In this work we have conjugated the lipidic amino acid with a fluorescent polar group (7-nitrobenz-2-oxa-1,3-diazol-4-yl), to mimic drug conjugates, and its association with serum proteins and lipid bilayers was characterized. The results obtained indicate that conjugates of polar molecules with lipidic α-amino acid, via covalent attachment to the amine group, have a relatively high solubility in aqueous solutions due to their negative global charge. They bind to serum albumin with intermediate affinity and show a very high partition coefficient into lipid bilayers in the liquid-disordered state. The attachment of the polar group to the lipidic amino acid increased strongly the aqueous solubility of the amphiphile, although the partition coefficient into lipid membranes was not significantly reduced. Conjugation of polar drugs with lipidic amino acids is therefore an efficient approach to increase their affinity for biomembranes.



1. INTRODUCTION

Lipidic α-amino acids have a long hydrocarbon chain in their lateral chain being significantly more hydrophobic than the common α-amino acids found in proteins. They are considered non-natural, although α-amino acids with very long hydrocarbons in the lateral chain (28 and 29 C) have been encountered in some cnidarians.[1] The natural function of those lipidic α-amino acids is not known, but they have been found to have cytotoxic activity toward tumor cell lines.[1]

The presence of the two functional groups in the lipidic amino acids makes them very interesting molecules in the design of prodrugs with increased bioavailability. Depending on the charge of the pharmacologically active moiety and on the preferred global charge, the lipidic amino acid may be attached to the polar drug via the amine or carboxylic acid group. Additionally, the metabolic stability of the resulting prodrug may be designed to fulfill the pharmacologic requirements from the labile ester bond to more stable covalent bonds involving the amine group.[2] Covalent modification at the amine or carboxylic group will also lead to amphiphiles with distinct dipole moments. This property has been shown to influence the interaction of the amphiphile with biological membranes of distinct lipid composition[3,4] and may be a relevant tool in the design of ligands targeted toward specific cells or membrane pools within the cell. The amphiphilic nature of the lipidic amino acids and their biocompatibility makes them interesting candidates as surfactants in the food and pharmaceutical industries.[5]

The synthesis of non-natural amino acids has attracted special attention in recent years because they play an important role in the design and synthesis of new molecules with relevant properties.[6] The Strecker synthesis, which is considered the first multicomponent reaction, is a versatile and cheap method to synthesize racemic α-amino acids, where the aldehyde is one of the three starting components.[7] Nowadays, hydroformylation, a reaction catalyzed by organometallic complexes, is considered one of the best approaches to convert alkenes into higher value aldehydes.[8] So, the combination of these two reactions in a

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one-pot hydroformylation/Strecker was recently described by Eilbracht as an attractive synthetic tool for the preparation of α-amino nitriles, which are important amino acid precursors.[9]

Lipidic α-amino acids have been used previously as conjugates of very polar pharmacologically active molecules in an attempt to improve their bioavailability due to the increased hydrophobicity.[10−17] The results obtained show a significant increase in the blood concentration after oral administration,[12] in the rate of permeation through Caco-2 cell monolayers[14] and in the rate of cell uptake[12] for some drug conjugates. However, the overall trend is very difficult to interpret as it does not follow any predictable dependence on the structure of the lipidic amino acid.[10,12] This may be partially due to the existence of specific transporters and efflux pumps or even due to distinct rates of degradation by the cells. In addition, the interpretation of the results is hampered by the lack of a previous characterization of the physicochemical properties of the lipidic amino acids and their conjugates. In spite of the wide use of the different lipidic amino acids, mostly by Toth and co-workers, the aqueous solubility has been reported only for the glucose conjugate of α-aminotridecanoic acid.[15] The interaction of some drug conjugates of lipidic amino acids with DPPC bilayers has also been qualitatively evaluated experimentally and calculated using available software,[13,16] but surprisingly, the results from the two approaches have not been critically compared.

The rate of passive permeation through confluent cell monolayers is usually considered to be proportional to the hydrophobicity of the solute, in agreement with the so-called "Overton rule".[18] However, for very hydrophobic solutes, the rate of interaction with the membranes may become the rate limiting step in the overall permeation process, breaking down the premises of the Overton rule.[19−22] The aggregation behavior of the amphiphilic drug and its relative affinity for proteins in the aqueous media and for the cell biomembranes are additional parameters that may lead to a nonmonotonic dependence of the rate of permeation on the amphiphile hydrophobicity.[23] It is therefore very important to characterize the monomeric solubility of the lipidic amino acids and their drug conjugates as well as their interaction with serum proteins and biomembranes. The length of the hydrocarbon chain may also influence the location of the amphiphile in the lipid bilayer[24,25] with important consequences on the amphiphile acid−base equilibria and on the kinetic and equilibrium parameters for the interactions with lipid bilayers.[20]

In this paper we describe the efficient synthesis of a lipidic α-amino acid with 11 carbons in the hydrophobic lateral chain (α-aminotridecanoic acid) and its conjugation with the polar fluorescent group NBD (7-nitrobenz-2-oxa-1,3-diazol-4-yl). The solubility of the monomeric form and the aggregation behavior, in aqueous media at pH = 7.4, were studied as well as the interaction with serum albumin and lipid bilayers prepared from 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocoline (POPC). Qualitative information regarding the rate of interaction with the POPC lipid bilayers is also reported.

2. MATERIALS AND METHODS

2.1. Materials and Equipment. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ or CD_3OD solutions on a Bruker 300 spectrometer, operating at 300.13 MHz for ^{1}H and at 75.47 MHz for ^{13}C. MS was carried out on Micromass Q-TOF 2 spectrometer containing a Z-spray source, an electrospray probe, and an injection syringe. GC was carried out on Agilent-6890, equipped with capillary HP5 columns and a FID detector. $Rh(acac)(CO)_2$, xantphos, and 1-undecene were purchased from Sigma Aldrich. Dimethoxyethane, used in the catalytic experiments, was purified by reflux over sodium lumps/benzophenone, for 8 h, and then distilled. Steady state fluorescence measurements were performed on a Cary Eclipse fluorescence spectrophotometer (Varian) equipped with a thermostatted multicell holder. UV−vis absorption was performed on a Unicam UV530 spectrophotometer (Cambridge, U.K.). Calorimetry titrations were performed on a VP-ITC instrument from MicroCal (Northampton, MA, U.S.A.) with a reaction cell volume of 1410.9 μL at 25 °C. The injection speed was 0.5 μL s^{-1}, stirring speed was 459 rpm, and the reference power was 2 or 10 μcal s^{-1}. The average size of the LUVs and their zeta potential was measured on a Malvern Nano ZS (Malvern Instruments, Malvern, U.K.).

2.2. Catalytic Experiments. The NMR spectra of the α-lipidic amino acid, the NBD conjugate, and the synthesis intermediates are given in the Supporting Information.

2.2.1. General Procedure for One Pot Hydroformylation-Strecker Reaction. The catalyst $Rh(acac)(CO)_2$ (0.01 mmol) with the ligand xantphos (0.05 mmol) were placed in a glass-lined stainless steel autoclave, which was closed and purged with three cycles of vacuum and a CO/H_2 equimolar mixture. A solution of 1-undecene (5 mmol) in DME (8 mL) was added via cannula into the autoclave, which was pressurized again to 20 bar with a CO/H_2 equimolar mixture and then heated at 80 °C. After 44 h, the reactor was cooled to room temperature and a water solution (2 mL) of NH_4Cl (10 mmol) and NaCN (10 mmol) was added via inlet cannula. The reaction was kept at room temperature for 1.5 h and then at 50 °C for 20 h. Upon completion, the reaction mixture was washed with brine solution, extracted with diethyl ether, and the organic layer was dried (Na_2SO_4) and evaporated. The residue was purified by silica gel column chromatography starting with CH_2Cl_2 as eluent, and solvent polarity was increased during the process by adding ethyl acetate, CH_2Cl_2/ethyl acetate (2:1).

*2-Aminotridecanonitrile, **3**.* ^{1}H NMR ($CDCl_3$, 300 MHz), δ, ppm: 0.88 (t, 3H, CH_3), 1.19−1.38 (m, 16H, CH_2), 1.44−1.53 (m, 2H, CH_2), 1.82 (q, 2H, CH_2), 4.46 (t, 1H, C<u>H</u>NH_2CN). ^{13}C NMR, δ, ppm: 120.2 (CN), 61.3 (<u>C</u>HNH$_2$CN), 35.2−14.1 (C_{alkyl}).

2.2.2. General Procedure for Hydrolysis of 2-Aminotridecanonitriles. The appropriate aminonitrile (isolated from the one pot hydroformylation/Strecker reaction) was suspended in a concentrated HCl solution and stirred for 6 h at room temperature. The solid product was filtered and washed with water (2 × 50 mL). Finally, the amino acid was dissolved in methanol, and the pH value was adjusted to the isoelectric point with an aqueous ammonia solution. The solvent was evaporated and the product was dried over vacuum.

*2-Aminotridecanoic Acid, **4** (C_{13}AA).* ^{1}H NMR ($CDCl_3$, 300 MHz), δ, ppm: 0.90 (t, 3H, CH_3), 1.21−1.37 (m, 14H, CH_2), 1.42 (m, 2H, CH_2), 1.68 (m, 2H, CH_2), 1.77 (m, 2H, CH_2), 3.98 (t, 1H, C<u>H</u>NH_2COOH). ^{13}C NMR , δ, ppm: 180.7 (COOH), 72.7 (<u>C</u>HNH$_2$COOH), 35.9−14.5 (C_{alkyl}). HRMS (ESI): *m*/*z* 230.2115 (M + H^+), calcd for $C_{13}H_{28}NO_2^+$, 230.2109.

2.2.3. Conjugation of 2-Aminotridecanoic Acid with 7-Nitrobenz-2-oxa-1,3-diazol-4-yl. The lipidic amino acid was dissolved in dry chloroform with 20% methanol and excess sodium carbonate was added. NBD-Cl dissolved in the minimal amount of dry chloroform was added dropwise with constant

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stirring. The mixture was left in the dark, with stirring, for 6 h at room temperature. A small excess of amino acid was used with a concentration of amine ~ 10 mg/mL. The reaction mixture was purified using Si60 preparative TLC plates and $CHCl_3$/MeOH/CH_3COOH 80:20:1 as eluent. The product, NBD-C_{13}AA being the most intense yellow band with and a retention factor ~0.2, was extracted from the stationary phase using chloroform/methanol mixtures and further purified by preparative liquid chromatography using the stationary phase RP18 and acetonitrile/water 75:25 as eluent.

NBD Conjugate of 2-Aminotridecanoic Acid (NBD-C_{13}AA). ^{1}H NMR (CD_3OD, 300 MHz), δ, ppm: 0.89 (t, J = 3.6 Hz, 3H, CH_3), 1.20−1.40 (m, 18H, CH_2), 1.45 (m, 2H, CH_2), 3.67 (m, 1H, CHCOOH), 6.02 (d, J = 4.8 Hz, 1H, NHCCH), 8.46 (d, J = 4.8 Hz, 1H, $CCHNO_2$).

2.3. Solubility of the Lipidic Amino Acid. The critical aggregation concentration (CAC) of C_{13}AA was determined using the method developed in ref 26, based on the increase in the fluorescence quantum yield and shift to smaller wavelengths of *N*-phenyl-1-naphthylamine (NPN) upon association with preformed aggregates. The total concentration of NPN was 100 nM, and the concentrations of C_{13}AA in the aqueous buffer were changed between 0 and 100 μM, at 25 °C. The aqueous solutions of C_{13}AA were prepared by addition of a small aliquot of amphiphile dissolved in methanol (the final concentration of methanol was always 0.5% v/v) and the required amount of NPN dissolved in aqueous buffer with 0.5% methanol was added. Solutions were left to equilibrate for 2−4 h, and the fluorescent emission of NPN was measured using an excitation wavelength of 356 nm.

The solubility of the monomeric form of NBD-C_{13}AA in aqueous solutions, at pH = 7.4 and 25 °C, was assessed via deviations to linearity in the amphiphile absorption and/or fluorescence as a function of the total concentration of amphiphile. The necessary volume of a solution of the amphiphile in methanol was placed in a glass tube, the solvent was evaporated under a stream of nitrogen, and the required amount of aqueous solution was added. The solutions were allowed to equilibrate at 25 °C for 2−4 h and their absorption and fluorescence were measured. In all measurements, the absorption (plus scatter) was smaller than 0.1 at the excitation wavelength guaranteeing a linear fluorescence response and minimizing inner filter effects. The concentration of NBD-C_{13}AA in the methanol solutions was calculated assuming ε = 2.1 × 10^4 M^{-1} cm^{-1} and within the experimental uncertainty the same molar absorption coefficient was observed for the amphiphile dissolved in the aqueous buffer.

2.4. Binding of NBD-C_{13}AA to BSA. The equilibrium constant between NBD-C_{13}AA and BSA was obtained using the approach described before.[25] A small aliquot of NBD-C_{13}AA, dissolved in methanol, was added to aqueous solutions with different concentrations of BSA (final concentration of methanol equal to 0.5%) and the solutions were allowed to equilibrate for 1−2 h. The amount of fluorescent amphiphile bound to albumin was assessed via the increase in its fluorescence upon binding.

The characterization of the binding of C_{13}AA to BSA was attempted using ITC. However, the experiments were not doable due to the large heat of dilution of the protein (when adding BSA to the ligand) and the low solubility of the ligand (if the ligand was to be added to the protein).

2.5. Partition of NBD-C_{13}AA to POPC Bilayers Followed by Fluorescence Energy Transfer. POPC bilayers were in the form of large unilamellar vesicles (LUV) and contained diphenyl hexatriene (DPH) at a molar ratio of 1:500. A mixture of POPC and DPH in azeotropic mixture of chloroform and methanol (87:13, v/v) was allowed to equilibrate for 1 h and the solvent was evaporated by blowing dry nitrogen over the heated (blowing hot air over the external surface of the tube) solution. The film was left in a vacuum desiccator for at least 8 h at room temperature to eliminate traces of organic solvent and then hydrated with the aqueous buffer. The hydrated lipid was subjected to several cycles of gentle vortex/incubation at room temperature for at least 1 h, to produce a suspension of multilamelar vesicles that was then extruded in a water-jacketed extruder (Lipex Biomembranes, Vancouver, British Columbia, Canada) using a minimum of 15 passes, through two stacked polycarbonate filter (Nucleopore) with a pore diameter of 0.1 μm.[27] The radius of the different LUVs preparations was 53 ± 4 nm, as shown by dynamic light scattering with a volume distribution analysis. The final phospholipid concentrations in the LUVs was determined using a modified version of the Bartlett phosphate assay,[28] and DPH was quantified by its absorption at 355 nm assuming ε = 8.1 × 10^4 M^{-1} cm^{-1}.[29] NBD-C_{13}AA dissolved in the aqueous buffer was added to the DPH:POPC LUVs at a total concentration of NBD-C_{13}AA equal to 4 μM and lipid concentrations from 0 to 1 mM. The solutions were allowed to equilibrate for 2 to 4 h and DPH fluorescence was measured with a path length of 5 mm.

2.6. Partition of NBD-C_{13}AA to POPC Bilayers Followed by Isothermal Titration Calorimetry. The titration was performed with additions of 10 or 20 μL of the lipid solution per injection and the concentrations in the cell were calculated considering that overflow is faster than mixing.[30] Amphiphilic ligands may adsorb to some equipment parts, in particular to the filling syringe, reducing the amount of ligand available. To overcome this difficulty, after cleaning thoroughly with water, the equipment cell was rinsed with a solution with the same composition as the solution to be used in the experiment before filling with the required titration solution. All solutions were previously degassed for 15 min. The titrations were performed in aqueous solutions containing 0.15 M NaCl, 0.02% NaN_3, and 1 mM EDTA. Additionally the pH was controlled at 7.4 with 10 mM of HEPES, phosphate or TRIS.

For the experiments where the pH was controlled with HEPES or phosphate, the interaction parameters (partition coefficient and interaction enthalpy) were obtained from the best fit to the differential heat curve.[31] However, for the experiments performed in TRIS buffer, the global interaction enthalpy (partition *plus* buffer ionization) is very small and a conventional titration curve cannot be obtained with acceptable signal-to-noise ratios. The interaction enthalpy was therefore obtained from the cumulative heat obtained for the complete partition of the amphiphile to the membrane, after subtraction of the dilution heat obtained from the addition of the lipid to the buffer in the absence of amphiphile.

Some qualitative information on the rate of translocation of the lipidic amino acid was obtained using the uptake and release protocol.[32] The POPC LUVs loaded with amphiphile were prepared at a probe to lipid ratio of 1:20 via the addition of an aqueous solution of POPC at 1 mM in phosphate buffer to a film of the amphiphile. The solution was allowed to equilibrate for 24 h at room temperature with occasional vortex stirring.

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2.7. Zeta Potential and Size of POPC LUVs Loaded with NBD-C_{13}AA. POPC LUVs in aqueous HEPES buffer without NaCl were prepared following the procedure indicated above. Different concentrations have been prepared and incubated with NBD-C_{13}AA (at 4 or 18 μM) for 6 h at 25 °C. The LUV solutions were always monodisperse in both size and zeta potential, their average size was evaluated from the size distribution by volume assuming η = 0.89 cP[33] and a refractive index of 1.33.

The aqueous solution used was 10 mM HEPES at pH = 7.4 with NaN_3 0.02% and 1 mM EDTA, as in the previous sections, but without NaCl. This decrease in the ionic strength of the solution was required to allow the measurement of the zeta potential.

3. RESULTS AND DISCUSSION

3.1. Synthesis of α-Aminotridecanoic Acid. To synthesize α-amino acids from the corresponding aldehydes obtained via hydroformylation reaction it is imperative to optimize the regioselectivity of the reaction in order to enhance the formation of the linear aldehyde. Our recent study on the optimization of the synthesis of a set of α-amino acids via one pot hydroformylation-Strecker sequential reaction showed that the regioselectivity for linear aldehyde depends on the phosphine structure and also on the reaction conditions (pressure and temperature).[34] The catalytic system that showed the best performance was Rh/xantphos at 80 °C of temperature and 20 bar of pressure. Therefore, for α-aminotridecanoic acid synthesis, the catalytic precursor $Rh(acac)(CO)_2$ and xantphos have been introduced in the autoclave and, after air evacuation, 1-undecene (1) and toluene were introduced via cannula and finally the pressure and temperature were adjusted: step A. Aliquots were taken to acquire the end of the hydroformylation reaction before proceed to the subsequent in situ Strecker reaction: step B. After the end of the hydroformylation step (44 h), the linear aldehyde (2) was obtained in 94% of yield. In order to obtain the corresponding aminonitrile (3), the reaction vessel was cooled until room temperature and stoichiometric amounts of NaCN and NH_4Cl were added. The α-aminotridecanonitrile (3) achieved in 83% yield was isolated and subsequently transformed into the desired lipidic amino acid (4) via hydrolysis with HCl, Scheme 1. The α-aminotridecanoic acid (4) obtained in 73% yield was fully characterized by ^{1}H and ^{13}C NMR and MS analysis (the spectra are given in the additional materials).

3.2. Solubility of α-Aminotridecanoic Acid in the Aqueous Media. The aggregation behavior of α-aminotridecanoic acid (C_{13}AA) was studied via variation in the fluorescence of NPN due to partition to the lipidic amino acid aggregates.[26] The value obtained for the critical aggregation concentration, at 25 °C (HEPES 10 mM pH = 7.4, NaCl 150 mM, EDTA 1 mM, 0.02% NaN_3), was 11 ± 5 μM, typical results are shown in Figure 1. Due to the low value of the solubility encountered for the monomeric species, a small concentration of NPN had to be used (100 nM) leading to a rather low fluorescence intensity when dissolved in the aqueous solution ([C_{13}AA] ≤ 6 μM). At higher amphiphile concentrations ([C_{13}AA] ≥ 15 μM), soft aggregates are formed to which NPN partitions with a significant increase in the fluorescence quantum yield and a concomitant blue shift in the emission maxima. The critical aggregation concentration (CAC) was obtained from the intercept of the straight lines describing NPN fluorescence in the two regimes.[26] The standard deviation indicated above for the CAC reflects variations observed on the intercept between the two regimes for over 10 independent experiments. The solutions above the CAC were characterized by dynamic light scattering indicating a high polydispersity index with an average diameter that increases with the amphiphile concentration. Just above the CAC, the large majority of the amphiphile is in small aggregates with a diameter of 1−5 nm, compatible with the formation of small micelles. As the total concentration of amphiphile is increased, the average size of the aggregates increases being centered at 300 nm for [C_{13}AA] = 30 μM.

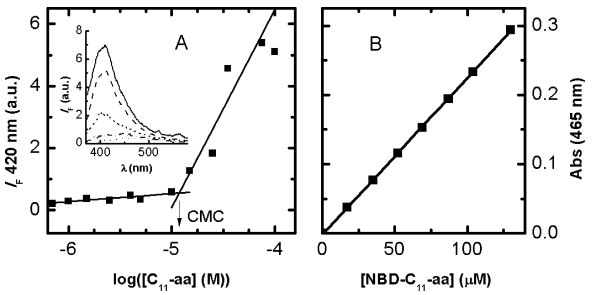
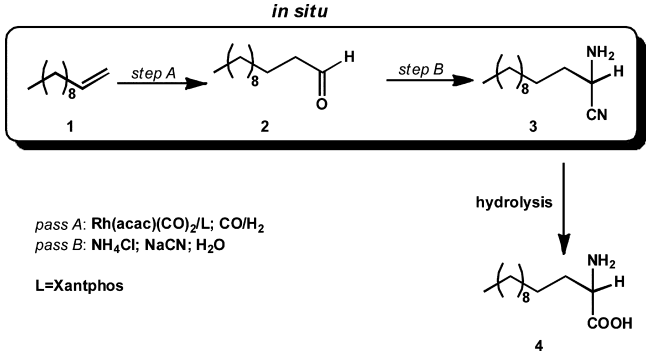


Figure 1. (A) Average of the NPN fluorescence emission between 410 and 430 nm in the presence of different concentrations of C_{13}AA at 25 °C in the aqueous buffer solution (HEPES 10 mM, pH = 7.4, NaCl 150 mM, EDTA 1 mM, and NaN_3 0.02%). The lines are the best fit of a straight line to each of the two regimes that intercept at the critical aggregation concentration of C_{13}AA. The inset shows the fluorescence spectra of the solutions at the amphiphile concentrations: 0 (·····), 10 (-··-), 25 (----), 35 (— —), and 75 μM (—). (B) Absorption at 465 nm of NBD-C_{13}AA at different concentrations in the aqueous buffer at 25 °C. The line is the best fit of a straight line.

The value encountered for C_{13}AA CAC is not easily compared with literature data because the aggregation behavior of lipidic amino acids has not been reported. The solubility of the monomeric form is significantly smaller than that observed for fatty acids with the same length in the hydrocarbon chain (CMC($C_{11}COO^-$) = 0.80 mM[35]) or for amino acid derived amphiphiles with a similar number of aliphatic carbons (CMC($C_{11}Arg^+$) = 5.8 mM,[36] CMC($C_{10}Cys^-$) = 0.42 mM[37]). This difference is a consequence of the neutral global charge of the lipidic amino acid used in this work that reduces significantly its solubility in the aqueous media as a monomer. Additionally, its zwitterionic nature and hydrogen bonding ability stabilizes the formation of large amphiphile aggregates.

Scheme 1



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3.3. Photophysics and Aqueous Solubility of α-Aminotridecanoic Acid Conjugated with a Polar, Drug-Like, Fluorescent Moiety (NBD-C_{13}AA). Lipidic amino acids have been attached covalently to polar drugs in an attempt to enhance their bioavailability.[10−13,16,38−41] In this work we have covalently attached the fluorescent moiety 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) to the α-amine group of compound **4** (NBD-C_{13}AA). The resulting secondary amine is expected to be a very weak base,[25] and therefore, in aqueous solutions at pH = 7.4, NBD-C_{13}AA has a negative charge. The aggregation behavior of the resulting amphiphile was first studied to characterize the concentration range where it is in the monomeric form and to guarantee that it is in this state at the concentrations used in the binding experiments. It was found that this amphiphile is soluble in the aqueous solution in its monomeric state up to a concentration of at least 130 μM, Figure 1B. Higher concentrations were not evaluated due to limitations in the amount of amphiphile available and on the method used. Nevertheless, those results show that this derivative is significantly more soluble than the starting amino acid as expected due to its global negative charge. This amphiphile is also much more soluble than the fatty amine with the equivalent hydrocarbon chain used by these authors in previous studies (CAC(NBD-C_{10}) = 0.05 μM) its solubility being comparable to that of fatty acids with similar hydrocarbon chains[35] and to the N-glucose conjugate of α-aminotridecanoic acid in the anionic form.[15]

The absorption spectra of the NBD conjugated amino acid in aqueous solution was significantly blue-shifted as compared with that of NBD fatty amines but the molar absorption coefficient at the maximum is very similar (~2.1 × 10^4 M^{-1} cm^{-1}). The fluorescence emission spectrum is red-shifted showing a significantly larger Stokes shift for the lipid amino acid conjugate, Figure 2. The fluorescence quantum yield of

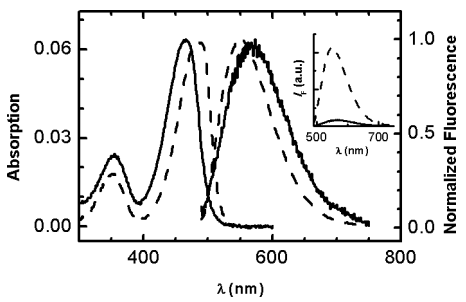

Figure 2. Absorption and normalized fluorescence emission spectra (when excited at 475 nm) of NBD-C_{13}AA (—) and NBD-C6 (----) in aqueous buffer at a concentration of 3 μM. The inset shows the fluorescence emission spectra without normalization highlighting the much smaller fluorescence quantum yield of NBD-C_{13}AA.

NBD-C_{13}AA in aqueous solutions is significantly smaller than that of NBD-C_n (~10 times), see inset in Figure 2 for comparison with NBD-C_6, in agreement with the red shift observed.

3.4. Binding of NBD-C_{13}AA to BSA. The rational underlying the conjugation of lipidic amino acids to polar drugs to enhance their bioavailability was the expected increase in the partition of the modified drug to biomembranes. Surprisingly, their partition coefficient between the aqueous media and lipid bilayers or their binding to blood proteins such as serum albumin has never been characterized. In this work we have studied the association of the NBD conjugate with the most abundant serum protein and the partition of both C_{13}AA and NBD-C_{13}AA between the aqueous phase and lipid bilayers.

The binding of NBD-C_{13}AA to bovine serum albumin (BSA) was characterized via the increased in the fluorescence quantum yield of NBD upon binding of the amphiphile to BSA,[25,42−44] Figure 3. Five independent experiments have been performed

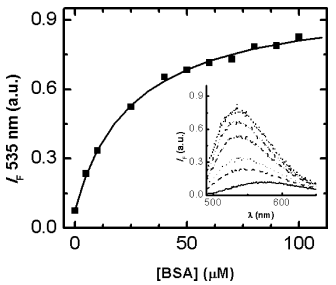

Figure 3. Binding of NBD-C_{13}AA to BSA in the aqueous buffer (HEPES 10 mM, pH = 7.4, NaCl 150 mM, EDTA 1 mM and NaN_3 0.02%) at 25 °C. The fluorescence intensity was normalized to that at total amphiphile binding and the line is the best fit of eq 1 with K_B = 3.8 × 10^4 M^{-1} and Φ_{AB}/Φ_A = 13. The inset show the fluorescence spectra of the solutions at [NBD-C_{13}AA] = 100 nM and different concentrations of BSA: 0 (—), 5 (− −), 10 (·····), 25 (- -), 40 (- ··), and 100 μM (----).

leading to K_B = 3.5(±0.6) × 10^4 M^{-1}. The results were analyzed with eq 1 that describes binding of the amphiphile (A) to a single binding site at a large excess of protein (B):

$$I_F = \Phi_A[A] + \Phi_{AB}[AB]$$

$$[A] = \frac{[A_T]}{1 + K_B[B]}; \quad [AB] = \frac{[A_T]K_B[B]}{1 + K_B[B]} \qquad (1)$$

where K_B is the binding constant and $[A_T]$ is the total concentration of amphiphile from which [A] is in the aqueous media and [AB] is bound to the protein. At the conditions used, total concentration of protein much higher than that of the amphiphile, the concentration of free protein [B] may be considered equal to its total concentration. The total fluorescence emitted by the solution is given by the concentration of amphiphile multiplied by a proportionality factor related with its fluorescence quantum yield considering both the probe in the aqueous media (Φ_A) and bound to the protein (Φ_{AB}).

3.5. Interaction of C_{13}AA and NBD-C_{13}AA with POPC Bilayers. *3.5.1. Equilibrium Partition Coefficient.* Partition of NBD-C_{13}AA to POPC bilayers was also studied but could not be followed directly via variations in the fluorescence quantum yield of NBD because this parameter was not significantly affected by interactions with the membrane. We have also tried to follow the transfer from BSA to POPC bilayers but the small fluorescence intensity from the amphiphile was masked by the large scatter from the liposomes.

The amount of NBD-C_{13}AA associated with the bilayer was evaluated from the fluorescence quenching of diphenyl hexatriene (DPH) previously associated with the POPC bilayer at a concentration of 0.2 molar %. The fluorescence quenching observed is due to nonradiative energy transfer from DPH to NBD when both probes are associated with the lipid bilayer as anticipated by the extensive overlap between DPH fluorescence and NBD absorption spectra, Figure 4A. Typical results obtained are shown in Figure 4B.

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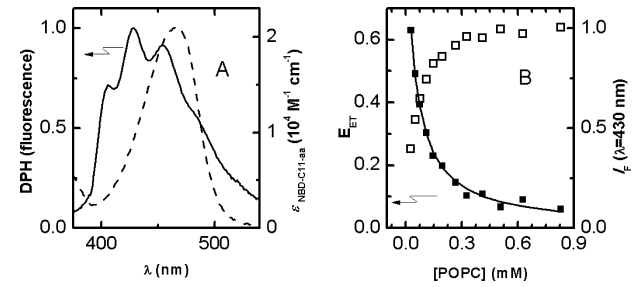

Figure 4. (A) Fluorescence emission spectra of DPH (—) and molar absorption coefficient of NBD-C_{13}AA (– –) in the presence of POPC LUVs. (B) Relative fluorescence intensity of DPH inserted in POPC bilayers due to energy transfer to NBD-C_{13}AA (□) and correspondent efficiency of energy transfer (■) calculated from eq 3 in the Supporting Information. The line is the best fit of eqs 1 and 2 in the Supporting Information for $R_e = 0$, with $K_P = 8.2 \times 10^4$ and $R_0 = 14$ Å.

The dependence observed on the efficiency of energy transfer (E_{ET}) with the concentration of lipid was analyzed with an analytical approximation for energy transfer in two dimensions.[45] Details of the model and fitting procedure may be found in the Supporting Information, together with a critical evaluation of the applicability of this model and accuracy of the parameters obtained given the information known regarding NBD and DPH photophysics[29,46,47] and the location and dynamics of NBD-amphiphiles in POPC bilayers.[20,24,25,47] The parameters obtained from the analysis of energy transfer results were strongly model dependent leading to a large uncertainty in the parameters obtained. Important qualitative information may still be obtained from those experiments as they undoubtedly show that the NBD moiety of the amphiphile is in close proximity to the POPC bilayer at the lipid concentrations used.

Quantitative information regarding the interaction of the amphiphiles with the POPC bilayer was obtained using isothermal titration calorimetry. A typical titration curve is shown in Figure 5A. The experimental results were analyzed with a model assuming simple partition, eq 2:

$$n_{C_{13}L} = n_{C_{13}T}\frac{K_P\overline{V}_L[L]}{1 + K_P\overline{V}_L[L]} \quad (2)$$

where $n_{C_{13}L}$ (i) is the number of moles of the lipidic amino acid (C_{13}AA or NBD-C_{13}AA) associated with the lipid bilayer, $n_{C_{13}T}$ is the total number of moles of ligand (in the aqueous phase *plus* associated with the lipid bilayer), K_P is the partition coefficient, [L] is the lipid concentration and $\overline{V}_L$ is the molar volume of the lipid (0.795 dm^3/mol for POPC[48]).

The heat evolved at each titration step ($q(i)$) was calculated from eq 3, where $V(i)$ is the volume of the lipid solution injected in the titration step i, V_{cell} is the volume of the calorimeter cell, q_{dil} is the heat of dilution of the POPC LUVs, and ΔH^0 is the enthalpy variation due to partition from the aqueous into the lipidic phase:

$$q(i) = \Delta H^0\left(n_{C_{13}L}(i) - n_{C_{13}L}(i-1)\left(1 - \frac{V(i)}{V_{cell}}\right)\right) + q_{dil} \quad (3)$$

The average values obtained for NBD-C_{13}AA from 10 titrations are $K_P = 5.0\ (\pm 0.9) \times 10^4$ and $\Delta H^0 = -0.9 \pm 0.2$ kJ/mol. We have also followed the partition of the lipidic amino acid without the NBD group (C_{13}AA) to the POPC bilayers using ITC and the average values obtained are $K_P = 6.2\ (\pm 1.1) \times 10^4$ and $\Delta H^0 = -1.5 \pm 0.7$ kJ/mol. In contradiction with the much lower solubility of the unlabeled lipid amino acid as compared with that of NBD-C_{13}AA, the affinity of C_{13}AA to the POPC bilayers is only slightly higher. This highlights the different forces that determine the two equilibria namely the energy difference between the amphiphile in the aqueous solution as monomer and as aggregates or inserted in the lipid bilayer. The strong interactions that are established between C_{13}AA molecules are responsible for its low solubility in aqueous solution but do not lead to significantly stronger interactions between the amphiphile and POPC as compared with those observed for NBD-C_{13}AA.

We call attention to the fact that the experimental conditions used in this section are not ideal, the lipid to amphiphile ratio at half titration is ~10, which is below the recommended minimum of 25.[31] The concentration of amphiphile could not however be decreased due to the very small enthalpy variation upon partition to the POPC membrane. At these conditions, the charge imposed by NBD-C_{13}AA in the lipid bilayer is not negligible and the correction for electrostatic effects in the model leads to a significant increase in the partition coefficient calculated. Only the observed partition coefficient is reported because the poor signal-to-noise ratio of the data does not support the use of complicated models that depend on a large number of parameters. Additionally, the ionization state of the amphiphile is not known accurately and may be dependent on the local concentration of amphiphile that changes throughout the titration, see section 3.5.4. We estimate an increase of 40% or up to 3 times in the intrinsic partition coefficient when a global charge of −0.2 or −1 is considered.

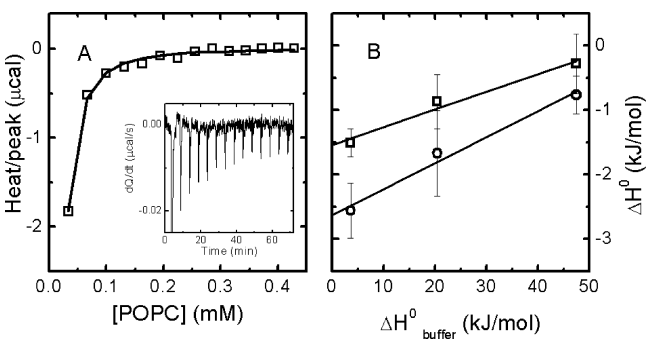

Figure 5. (A) Heat evolved due to the partition of NBD-C_{13}AA to POPC bilayers (□), in HEPES buffer, and best fit assuming simple partition and all lipid accessible to the amphiphile,[31] eqs 2 and 3, with $K_P = 4.6 \times 10^4$ and $\Delta H^0 = -0.9$ kJ/mol. The inset shows the corresponding thermogram. The amphiphile was initially at a concentration of 13 μM in the cell aqueous solution and 20 μL aliquots of POPC LUVs at 2.5 mM lipid were added. (B) Dependence of the observed enthalpy variation associated with partition to POPC bilayers on the ionization enthalpy of the pH buffer used (phosphate, HEPES or TRIS) for NBD-C_{13}AA (□) and C_{13}AA (○). The lines are the linear best fit to the experimental results indicating an intrinsic partition enthalpy variation (ΔH_P^0) of −1.5 ± 0.2 (−2.6 ± 0.3) kJ/mol and 0.03 (0.04) H^+ transferred from the buffer to NBD-C_{13}AA (C_{13}AA) upon partition.

3.5.2. Enthalpy of the Interaction. The enthalpy variation observed upon partition of the amphiphiles to the lipid bilayer is a result of both (i) the interactions established in the aqueous media and inserted in the bilayer and (ii) transfer of H^+

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between the buffer and the amphiphile as a result of variations in the amphiphile ionization state upon insertion in the membrane. The partition experiment was repeated in aqueous solutions of pH buffers with different ionization enthalpies ($\Delta H^0_{ionization}$ (phosphate) = 3.6 kJ/mol, $\Delta H^0_{ionization}$ (HEPES) = 20.4 kJ/mol and $\Delta H^0_{ionization}$ (TRIS) = 47.45)[49] to separate the contributions from partition and changes in the ionization state. The results obtained are shown in Figure 5B and indicate that the protonated state of the lipidic amino acid is stabilized by ~5% upon partition to the POPC membrane. The enthalpy variation due to partition to the bilayer is obtained from the intercept (at zero ionization enthalpy variation) and is −1.5 ± 0.2 kJ/mol for NBD-C_{13}AA and −2.6 ± 0.3 kJ/mol for C_{13}AA. Those values are significantly smaller than the ones obtained for most amphiphiles with similar hydrocarbon chains (partition between water and lipid bilayers in the liquid disordered state at 25 °C: ΔH^0_P (SDS) = −18 kJ/mol,[31,50] ΔH^0_P (NBD-C_n) = −15 kJ/mol,[25] ΔH^0_P ($C_{11}COO^-$) = −10 kJ/mol[35]) but small interaction enthalpies have also been reported (ΔH^0_P (DTAC) = −4.1 kJ/mol,[50] ΔH^0_P (lysoC$_{10}$PC) = −0.6 kJ/mol[35]).

The parameters obtained for the solubility of the amphiphiles and their association with BSA and POPC bilayers are collected in Table 1.

Table 1. Parameters Obtained for the Solubility of the Lipidic Amino Acid (C_{13}AA) and Its NBD Derivative (NBD-C_{13}AA) and for Their Interaction with BSA and POPC Bilayers at 25 °C

		binding to BSA	partition to POPC	
	CAC (μM)	K_B (10^4 M^{-1})	K_p (10^4)[a]	ΔH^0_P (kJ/mol)
C_{13}AA	11 ± 5		6.2 (±1.1)	−2.6 (±0.3)
NBD-C_{13}AA	≥130	3.5 (±0.6)	5.0 (±0.9)	−1.5 (±0.2)

[a]The indicated partition coefficient was obtained by ITC at zero enthalpy of ionization for the buffer, electrostatic effects were not considered.[31]

3.5.3. Rate of Translocation of C_{13}AA through POPC Bilayers. Qualitative information regarding the rate of translocation of C_{13}AA through POPC bilayers was obtained using the Uptake and Release protocol.[32] This method relies on differences between the total lipid (and amphiphile) concentration and the effective concentration ([L]* and [C_{13}]*) that is available for the equilibration of the amphiphile between the aqueous and lipidic phases during the time of the experiment and allows the calculation of the equilibration factor (γ), eq 4.[19,32]

$$[\mathrm{L}]^*(i) = [\mathrm{L}]^*(i-1)\left\{1 - \frac{V(i)}{V_{cell}}\right\} + \gamma\left\{[\mathrm{L}]^{syr}\frac{V(i)}{V_{cell}}\right\}$$

$$n^*_{C_{13},T}(i) = n^*_{C_{13},T}(i-1)\left\{1 - \frac{V(i)}{V_{cell}}\right\} + \{\gamma[C_{13}]^{syr}_L + [C_{13}]^{syr}_W\}V(i) \quad (4)$$

The equilibration factor was obtained through the global best fit of eqs 3 and 4, with the moles of amphiphile in the lipidic phase being calculated from eq 2, to the results from the uptake and release experiments.[19] Typical results are shown in Figure 6.

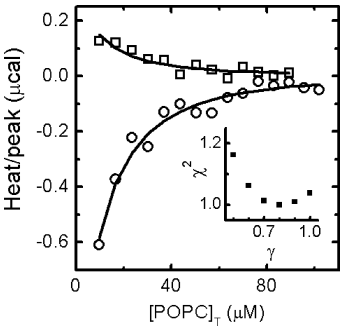

Figure 6. Heat evolved due to the addition of POPC LUVs to 5 μM C_{13}AA in phosphate buffer (○) and due to the dilution of C_{13}AA/POPC 1:20 into phosphate buffer (□), as a function of the total concentration of POPC in the cell. The lines are the global best fit with $K_P = 6.0 \times 10^4$, $\Delta H^0 = -2.5$ kJ/mol, and $\gamma = 0.8$. The inset shows the dependence of the global fit squared residues as a function of the fraction of lipid accessible (γ).

The value obtained for γ in the experiments with the partition of C_{13}AA is 0.7 ± 0.1. The equilibration factor has a clear physical meaning for values equal to 0.5 (only the lipid in the outer monolayer is accessible to the amphiphile in the uptake experiment and only the amphiphile in the outer monolayer is able to equilibrate with the aqueous media in the release experiment; slow translocation) and 1 (the amphiphile is able to equilibrate between the aqueous media and all the lipidic phase (outer and inner monolayer); fast translocation). Intermediate values are obtained when the amphiphile translocates only partially during the time of the experiment (200 s *per* peak in this work). The value obtained for C_{13}AA, γ = 0.7, indicates that the rate of translocation is within the range 10^{-2} to 10^{-3} s^{-1}.

This approach could not be followed for the case of NBD-C_{13}AA due to the very small enthalpy variation upon partition to the POPC bilayer. The translocation of this amphiphile is however expected to be fast, when compared to the characteristic time for the titration experiment ($\tau \approx 10$ s), given that fatty acids translocate through bilayers in the liquid disorder state with characteristic times smaller than 2 s[51−53] and that the neutral amphiphile NBD-C_{12} shows a characteristic time for translocation equal to 3 s.[20] Additionally, we find that most carboxylic acid is in the neutral form when NBD-C_{13}AA is inserted in the lipid bilayers, section 3.5.4.

We have recently shown that, for amphiphiles that translocate in the time scale of the titration experiment, quantitative information may be obtained from the time dependence of the heat evolved during each titration step.[19] Unfortunately, the very small interaction enthalpy variation upon partition of this amphiphile to the POPC bilayer leads to a low signal-to-noise ratio that makes this analysis unfeasible.

3.5.4. Effect of the Amphiphiles on the Charge and Size of the POPC LUVs. We have measured the zeta potential (ζ) of the LUVs at different lipid-to-NBD-C_{13}AA ratios to have further insight regarding the ionization state of the amphiphile when inserted in the POPC bilayer and complement the data obtained in section 3.5.2. The size of the liposomes was also measured to inspect for eventual LUVs disruption at the relatively high local concentration of amphiphile used in the previous sections. Typical results are shown in Figure 7.

The surface concentration of NBD-C_{13}AA was calculated from the partition coefficient obtained by ITC. This allows the prediction of the charge density at the surface of the bilayer as a

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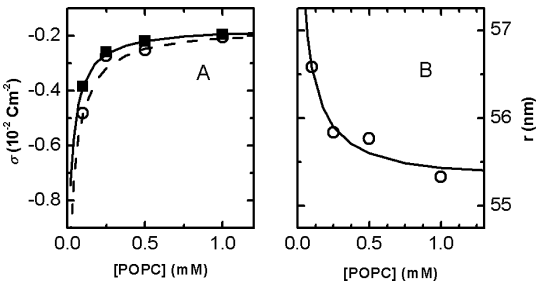

Figure 7. Surface charge density (A) and liposome radius (B) of POPC LUVs as a function of the lipid concentration, for a total concentration of NBD-C_{13}AA equal to 4 (■) or 18 (○) μM previously incubated with the liposomes for 6 h. The lines are the best fit assuming the partition coefficient obtained by ITC, $K_P = 5 \times 10^4$, see text for the model used and the values of the other parameters required.

function of the fractional charge of the amphiphile when inserted in the bilayer. From the measured zeta potential it is possible to calculate the surface potential[54] and this allows in turn the calculation of the surface charge density.[19,31] It should be noted that the calculation of the surface potential from the zeta potential requires the knowledge of the distance between the surface of the membrane and the plane of shear. We have used 2 Å for this distance as is generally accepted,[54] although this value is not well supported by experimental or theoretical work and is therefore subject to a very large uncertainty.

The minimization of the square deviation between the surface charge density calculated from (*i*) the amphiphile charge and partition coefficient and (*ii*) the surface potential allows the finding of the best partition coefficient and fractional charge of the amphiphile in the membrane. The lines shown in Figure 6A are the best fit assuming the partition coefficient from ITC, and lead to a fractional charge for NBD-C_{13}AA of −0.29 and −0.11 for a total concentration equal to 4 and 18 μM, respectively. The sensitivity of the squared residuals on the parameter values is however quite shallow down to $K_P = 2 \times 10^4$ where the calculated fractional charge is −0.4 and −0.15 for a total concentration of NBD-C_{13}AA equal to 4 and 18 μM, respectively. Those results show that, at the low ionic strength used in these experiments, the global charge of NBD-C_{13}AA inserted in the POPC bilayer is negative but deviates significantly from −1. It also shows that the local concentration of amphiphile affects significantly its ionization as expected from the surface potential generated by the charged amphiphile. The global charge of the amphiphile is expected to be somewhat more negative at the higher ionic strength used in the previous sections that are representative of physiological fluids.

The size of the POPC LUVs incubated with 18 μM of NBD-C_{13}AA was also measured. The samples were all monodisperse and the average size is shown in Figure 6B. A small but significant increase is observed as the concentration of POPC is decreased (larger ratio of ligand to lipid) in agreement with the insertion of the amphiphile in the bilayer. The radius of the liposome may be calculated from the total number of amphiphile (n_{AL}) and phospholipid (n_L) molecules in the liposome and their respective areas (A_L and A_{AL}), eq 5. The line shown in plot B is the best fit of eqs 5 and 2 assuming 63 Å^2 for the area of POPC[55] and the partition coefficient obtained by ITC. The area occupied by NBD-C_{13}AA when inserted in the bilayer was obtained as an adjustable parameter being 25 Å^2.

$$r_{LUV} = \sqrt{\frac{A^o_{LUV}}{4\pi}}; \quad A^o_{LUV} = \frac{A_L n_L + A_{AL} n_{AL}}{2} \tag{5}$$

4. CONCLUSIONS

The multicomponent sequential reaction based on the hydroformylation of 1-undecene followed by the classic Strecker reaction using the Rh/xantphos as catalysts revealed to be a selective and green approach to synthesize lipid aminoacids, namely α-aminotridecanoic acid.

The aqueous solubility of the lipidic amino acid covalently modified with the NBD group (NBD-C_{13}AA) is comparable to that of fatty acids with similar hydrocarbon chains[35] and to N-glucose derivatives of C_{13}AA[15] being much higher than that of the unconjugated lipidic α-amino acid. The NBD conjugate is much more soluble in aqueous media than NBD-fatty amines with the same alkyl length being similar to those with 4 or 6 carbons in the alkyl chain [NBD-C_4 and NBD-C_6 (some of the numbers indicated in Table 1 of publication 25 are incorrect due to typing errors; for NBD-C_6, the value encountered experimentally for the CAC was 1.3 (±0.1) × 10^{-5} M and for NBD-C_8, CAC = 9.1 (±1.2) × 10^{-7} M and K_B = 5.2 ± 0.3 × 10^5 M^{-1}, as shown in the reference figures)],[25] indicating that the longer alkyl chain in the lipidic amino acid is compensated by the presence of the carboxylic group in the polar region of the molecule. In agreement with those results, the value obtained for the binding constant between the lipidic amino acid and BSA is between those encountered for NBD-C_4 and NBD-C_6.[25] However, the partition of the lipidic amino acid to the POPC bilayers is much more efficient than for the case of NBD-C_4 or NBD-C_6 being comparable to that obtained for NBD-C_8. This difference is a result of a more favorable interaction between the lipidic amino acid derivative and the lipid bilayer and highlight the difficulty in the prediction of the bioavailability of a given amphiphile from a single estimator of their global hydrophobicity (such as its solubility in the aqueous media, binding to serum proteins, partition between organic solvents and water or partition to lipid bilayers). However, the complete characterization of the interaction of all drugs with all binding agents in the blood and cell membranes is not feasible. It is therefore of high importance the detailed characterization of some selected amphiphilic drugs to allow the establishment of rules that may be used to predict the behavior of unrelated drugs.

The parameters obtained indicate that the conjugation of drugs with lipidic amino acids is a very promising approach to increase their bioavailability because a good aqueous solubility is maintained while the affinity for biological membranes is strongly increased. We have also observed a relatively weak binding to serum proteins, which further improves their potential use to increase the bioavailability and pharmacokinetics of polar drugs.

■ ASSOCIATED CONTENT

Supporting Information

Details on the synthesis of C_{13}AA and NBD-C_{13}AA, as well as the characterization of final products and intermediates by ^{1}H NMR, are given. The model used to analyze quantitatively the partition of NBD-C_{13}AA to POPC bilayers via energy transfer from DPH is also given. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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