Characterization of daptomycin oligomerization with perylene excimer fluorescence: Stoichiometric binding of phosphatidylglycerol triggers oligomer formation

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Abstract

Daptomycin is a lipopeptide antibiotic that binds to and depolarizes bacterial cell membranes. Its antibacterial activity requires calcium and correlates with the content of phosphatidylglycerol in the target membrane. Daptomycin has been shown to form oligomers on liposome membranes. We here use perylene excimer fluorescence to further characterize the membrane-associated oligomer. To this end, the N-terminal fatty acyl chain was replaced with perylene-butanoic acid. The perylene derivative retains one third of the antibacterial activity of native daptomycin. On liposomes containing phosphatidylcholine and phosphatidylglycerol, as well as on *Bacillus subtilis* cells, the perylene-labeled daptomycin forms excimers, which shows that the N-terminal acyl chains of neighboring oligomer subunits are in immediate contact with one another. In a lipid bicelle system, oligomer formation can be titrated with stoichiometric amounts of phosphatidylglycerol. Therefore, the interaction of daptomycin with a single molecule of phosphatidylglycerol is sufficient to trigger daptomycin oligomerization.

Introduction

Daptomycin is a cyclic lipopeptide that consists of 13 amino acid residues, including one kynurenine residue and several other non-standard amino acids, and a decanoyl fatty acyl residue attached to the exocyclic N-terminal tryptophan residue (see Figure 1). It is produced by the soil bacterium *Streptomyces roseosporus*. Daptomycin is used in the treatment of infections by Gram-positive bacteria such as enterococci and staphylococci, including strains that are resistant to penicillin, methicillin and vancomycin [1, 2]. Daptomycin binds and depolarizes the bacterial cytoplasmic membrane [3]. Its activity requires calcium and correlates with the target membrane's content of phosphatidylglycerol (PG); increased conversion of PG to lysyl-PG confers partial resistance to bacterial cells [4, 5].

Using FRET between the kynurenine residue of unlabeled daptomycin and a fluorescently labeled derivative of daptomycin, we have shown before that daptomycin forms oligomers on liposome membranes [6]. Oligomerization occurs under conditions that resemble those required for its antibacterial activity, suggesting that the oligomer is involved in the bactericidal effect.

In this study, we have used excimer fluorescence in order to further characterize the membrane-associated daptomycin oligomer. An excimer, or excited dimer, is a complex that forms from one fluorophore molecule that is in the excited state and another one in the ground state [7]. Since this requires direct contact between two fluorophore molecules, the detection of excimer fluorescence imposes a more stringent constraint on the distance of the interaction than that implied by the observation of FRET.

The fluorophore most widely used to study excimer fluorescence is pyrene, whose excimer and monomer spectra are well separated. However, when pyrene was incorporated into daptomycin, we observed that most pyrene fluorescence was lost due to energy transfer to kynurenine. We therefore chose to incorporate perylene, which emits at longer wavelengths [8] and therefore

should not engage in FRET to kynurenine.

A prior study has shown that substitutions of the N-terminal decanoyl moiety of daptomycin are quite compatible with its antibacterial activity, as long as the introduced substituents retain an overall hydrophobic character [9]. Pyrene and perylene are very hydrophobic molecules, and accordingly they were introduced into daptomycin by replacing the decanoyl chain with pyrene-butanoic acid and perylene-butanoic acid, respectively. The derivatives retained antibacterial activity, albeit at a slightly reduced level in the case of perylene-daptomycin. Nevertheless, oligomer formation of perylene-daptomycin was clearly evident through excimer fluorescence, under conditions that mirrored those use in the previous FRET experiments. This result indicates that the fatty acyl residues of adjacent subunits of the membrane-associated daptomycin oligomer are within immediate reach of one another.

In a lipid bicelle system, the excimer fluorescence could be titrated with phosphatidylglycerol. Conversion of monomer fluorescence to excimer fluorescence approached saturation at an equimolar ratio of phosphatidylglycerol to daptomycin. Therefore, a single molecule of phosphatidylglycerol is sufficient to convert daptomycin to a conformation that is competent for oligomerization.

Our study provides significant additional insight into the process of daptomycin oligomerization. At the same time, it demonstrates that perylene excimer fluorescence is useful as a probe in the experimental study of biochemical systems.

Materials and Methods

Synthesis of perylene-daptomycin. To a suspension of deacylated daptomycin bearing a Boc protecting group on the ornithine residue (120 mg, 0.077 mmol; generously provided by Jared Silverman of Cubist Inc.) in dry dimethylformamide (0.5 mL) was added 4-(3-perylenyl)butanoic acid succinimidyl ester (see supporting information; 0.035g, 0.08 mmol). The mixture was stirred for 24 h and then concentrated by high vacuum rotary evaporation. The residue was dissolved in water and lyophilized. The lyophilized powder was dissolved in an ice cold solution of trifluoroacetic acid (TFA) containing 2% thioanisole, and the resulting solution was stirred for 15 minutes at 0 ºC (ice bath). The mixture was concentrated using a high vacuum rotary evaporator, keeping the heating bath below 40 ºC. The residue was dissolved in water and lyophilized. The resulting crude powder was purified by semi-preparative reversed phase HPLC employing a linear gradient starting with 95% water (0.1% TFA)-5% acetonitrile to 100 % acetonitrile over 50 minutes (t_r=34 min). Fractions containing the desired product were pooled and concentrated using a high vacuum rotary evaporator keeping the heating bath below 40 ºC. The resulting residue was lyophilized to give 0.047 g (34% yield) of perylene-daptomycin as a yellow powder. $^-$ ESIMS: 1784.76 $(M-H)^{-1}$ (Figure S1). The analytical reversed-phase HPLC chromatogram of the purified material, using a linear gradient starting with 95% water (0.1% TFA)-5% acetonitrile to 100 % acetonitrile over 50 minutes is shown in Figure S2.

Synthesis of pyrene-daptomycin. This compound was prepared using the same procedure as that described above for perylene-daptomycin using 0.0081 g (0.021 mmol) 1-pyrenebutanoic acid succinimidyl ester and 0.030 g (019 mmol) deacylated daptomycin bearing a Boc protecting group on the ornithine residue. The crude product was purified by HPLC as described above for perylene-daptomycin (t_r=28 min). Fractions containing the desired product were pooled and concentrated and the resulting residue lyophilized to give 17 mg (51% yield) of pyrene-

daptomycin as a white powder. $^{-}$ESIMS: 1734.75 $(M-H)^{-1}$ (Figure S3). The analytical reversed-phase HPLC chromatogram of the purified material using the conditions described above for perylene-daptomycin is shown in Figure S4.

Antibacterial activity test for pyrene- and perylene-daptomycin. The two labeled derivatives as well as native daptomycin were tested for antibacterial activity by broth dilution. LB broth was supplemented with calcium (5 mM), and the daptomycin derivative in question was added at concentrations of 10, 5, 3, 2, 1.5, and 1 μg/ml. Each tube was inoculated with *Bacillus subtilis* ATCC 1046 and incubated overnight at 37 ºC. Growth inhibition was evaluated visually by turbidity. Growth controls without antibiotic were prepared for comparison.

Preparation of large unilamellar vesicles (LUV). 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (DMPG), 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphoethanol (POPE), 1,2-dioleoyl-sn-glycerol-3-phospho-(1-rac-glycerol) (DOPG), and 1,3-bis[1,2-dioleoyl-sn-glycero-3-phospho]-sn-glycerol (tetra-oleyl-cardiolipin; TOCL) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). The required amounts of DMPC and DMPG, or of POPE, DOPG and cardiolipin, were weighted, dissolved in chloroform and transferred to a round-bottom flask. The solvent was evaporated with nitrogen and the resulting lipid films further dried under vacuum for three hours. The lipids were then suspended in HEPES 20 mM, pH 7.4, NaCl 150 mM (HBS). Finally, the suspension was extruded 10-15 times through 100 nm polycarbonate filters, using a nitrogen-pressurized extrusion device [10]. The liposomes were employed in the fluorescence experiments at a final concentration of 250 μM total lipid.

Fluorescence measurements. Emission spectra were acquired on a QuantaMaster 4 spectrofluorimeter (Photon Technology International, London, ON). Pyrene fluorescence was excited at 340 nm and the emission recorded from 360 to 600 nm. Perylene fluorescence was

excited at 430 nm and the emission recorded from 440 to 600 nm. Bandpasses for excitation and emission were between 2 and 5 nm, depending on the sample concentration. Emission spectra were corrected for wavelength-dependent instrument response using a quinine sulfate standard and a tabulated normalized quinine spectrum listed in [7].

Fluorescence lifetime measurements were performed on a FluoTime 100 Lifetime spectrometer (PicoQuant, Berlin, Germany), using diode laser light sources emitting at 370 nm and at 440 nm for pyrene and perylene, respectively. The emission was isolated using 460±10 nm or 560±10 nm bandpass filters (Melles-Griot, Brossard, QC). Experimental decays were numerically fitted with two or three exponentials, with resulting χ^2 values typically between 1.1 and 1.3. From these fitted exponential components, average lifetimes were calculated according to the equation

$$\langle \tau \rangle = \frac{\sum \alpha_i \tau_i^2}{\sum \alpha_i \tau_i}$$

were α_i represents the amplitude at time zero and τ_i the lifetime of the i^{th} component.

All measurements were acquired at room temperature. The concentration of calcium was 200 mM with liposomes consisting of PC only. With all other samples, the calcium concentration was 5 mM unless stated otherwise.

Deconvolution of perylene excimer and monomer spectra. Perylene fluorescence decays were measured at 560±10 nm on DMPC/DMPG membranes, both with pure perylene-daptomycin and with a mixture of perylene-daptomycin and a fivefold excess of unlabeled daptomycin. The decays were fitted to a two-exponential model. The fractional emission intensities of the monomer and the excimer at 560 nm were obtained from these data according to

$$F_{monomer,560} = \frac{\alpha_1 \tau_1}{\alpha_1 \tau_1 + \alpha_2 \tau_2} \quad \text{and} \quad F_{excimer,560} = 1 - F_{monomer,560}$$

From the spectrum of perylene-daptomycin only, *P*, and the spectrum of the mixture of perylene-

daptomycin with unlabeled daptomycin, *U*, the pure excimer spectrum, *E*, was calculated as

$$E = P - aU$$

with

$$a = \frac{F_{P,monomer,560}\, I_{P,560}}{F_{U,monomer,560}\, I_{U,560}}$$

where $I_{P,560}$ and $I_{U,560}$ denote the intensity of *P* and *U* at 560 nm, respectively. The pure monomer spectrum *M* was obtained analogously. After normalizing *E* and *M* to the same quantity of molecules, the quantum yield of the excimer, relative to that of the monomer, was calculated from the ratio of the areas of the two normalized spectra.

Titration of perylene excimer fluorescence with phosphatidylglycerol. To a solution of 100 μM perylene-daptomycin, 1 mM Ca^{++} and 20 mM DHPC in HBS, a mixture of DMPC and DMPG (molar ratio, 4:1) was added in a stepwise fashion. After each addition, the mixture was incubated for 2 minutes before acquisition of the perylene emission spectrum. Repeated scans showed that this short incubation sufficed to reach equilibrium. From each spectrum, the ratio of the emission intensities at 521nm and 480 nm was calculated, and the increment was plotted as a function of the molar ratio of DMPG to perylene-daptomycin.

Results

Synthesis and purification of pyrene- and perylene-daptomcyin. The synthetic procedure, as well as mass spectrometry and chromatography data on the product are given in the supplementary information file. The structures of pyrene- and perylene-daptomycin are shown in Figure 1.

Antibacterial activity of pyrene- and perylene-daptomycin. The antibacterial activity of pyrene- and perylene-daptomycin against *Bacillus subtilis* strain ATCC 1046 was determined by broth dilution, using native daptomycin as a reference. The minimum inhibitory concentration of perylene-daptomycin was 3 μg/ml, compared to 1 μg/ml for native daptomycin and pyrene-daptomycin. Therefore, the specific activity of perylene-daptomycin is approximately 3 times lower than that of native daptomycin and of pyrene-daptomycin.

Fluorescence of pyrene-daptomycin. Figure 2 shows the emission spectra of pyrene-daptomycin in solution and on liposomes consisting of phosphatidylcholine (PC) and phosphatidylglycerol (PG). Pyrene monomer fluorescence, which is apparent in the small peaks to both sides of 380 nm, is very low in both samples. There is a broad peak around 460 nm that greatly increases after incubation with the liposomes, on which daptomycin has been shown to form oligomers.

While oligomer formation should be accompanied by excimer formation, the latter should be accompanied by a decrease of monomer emission, which however is low to begin with and does not decrease any further. Furthermore, the peak at 460 nm has an average excited state lifetime of approximately 3.5 nanoseconds (data not shown), whereas the typical lifetimes of pyrene excimers are much longer. On the other hand, the observed lifetime is very similar to that found previously for the kynurenine residue of daptomycin [6].

The monomer emission spectrum of pyrene significantly overlaps the absorption spectrum of the kynurenine residue, which should cause fluorescence energy transfer from excited pyrene to kynurenine. The increase in the sensitized kynurenine emission upon membrane binding mirrors

that of directly excited kynurenine fluorescence reported earlier [11].

In sum, because of spectral overlap between pyrene emission and kynurenine absorption, pyrene-daptomycin does not produce experimentally useful excimer fluorescence.

Observation of perylene-daptomycin excimer fluorescence. The emission spectrum of perylene-daptomycin in solution resembles that of monomeric, free perylene in hexane [8], with three distinct peaks between 450 and 520 nm (Figure 3A). A similar spectrum is obtained when perylene-daptomycin is incubated with DMPC membranes (Figure 3B). In contrast, on membranes composed of DMPC and DMPG, as well as on bacterial cells, the shape of the spectrum changes significantly (Figure 3C, D). The two leftmost peaks are greatly decreased, and a new, broad peak emerges that overlaps the monomer fluorescence. While the new peak is centered around 525 nm and therefore is blue-shifted relative to the excimer of free perylene [8], its shape nevertheless is suggestive of excimer fluorescence. This interpretation is corroborated by time-resolved fluorescence measurements. Figure 4 shows the fluorescence decays measured at 560 nm of perylene-daptomycin on PC liposomes (A) and on PC/PG liposomes (B). Both decays can be fitted quite well with a two-exponential model (see fit parameter values in the figure), and the lifetime parameters obtained from these fits are similar to those of perylene monomers and excimers reported earlier [8]. Note that the pre-exponentials are different between the two membranes, which reflects a higher prevalence of excimers on the PC/PG membranes. Also, the fitted fluorescence lifetime of the putative excimer component is shorter in PC than in PC/PG membranes.

Excimer fluorescence only occurs when a monomer in the excited state binds directly to a another monomer that is in the ground state. It should therefore be greatly facilitated by the formation of stable oligomers, since within these the perylene moieties should remain close to one another throughout. On the other hand, a mixture of perylene-daptomycin with an excess of unlabeled

daptomycin should produce hybrid oligomers, within which the perylene-labeled molecules should become separated from one another by intervening unlabeled molecules. This should lead to increased monomer fluorescence and decreased excimer fluorescence. Figure 5 shows that this is indeed the case. It also shows that, on PC membranes, the ratio of excimer fluorescence to monomer fluorescence is not affected by the addition of unlabeled daptomycin. This is in line with the assumption that stable oligomers do not form on these membranes, and that excimer fluorescence arises by diffusional collision of individual perylene-daptomycin molecules only.

Very similar spectra and fluorescence lifetimes were obtained on liposomes consisting of POPE, DOPG and TOCL, which are considered models of the cytoplasmic membranes of bacteria [12, 13]. This is in line with prior observations [6].

Quantitative evaluation of excimer fluorescence. By combining lifetime data with steady state spectra, we can obtain an approximation of the spectrum of the excimer, as well as an estimate of its quantum yield relative to that of the monomer. The calculation, which is detailed in the Methods section, yields the monomer and excimer spectra shown in Figure 6. The quantum yield of the excimer, relative to that of the monomer, is 0.35.

On PC/PG membranes, the observed excimer emission corresponds to > 90% of all excitation events. If we neglect excimer emission due to collisional encounters of the few remaining monomers, we can infer that more than 90% of all daptomycin molecules are now located in oligomers. Considering that some excited perylene molecules will emit monomer fluorescence even when placed in the vicinity of other perylene molecules, the extent of oligomer formation may well be even higher.

At an oligomerization efficiency of 90%, the ratio of emission at 521 nm to that at 482 nm is 1.35 (Figure 3C). This ratio is 0.33 for PC liposomes (Fig. 3B), on which perylene-daptomycin emits mostly as a monomer. On bacterial cells (Fig. 3D), it is 1.16, which shows that most of the cell-

bound perylene-daptomycin have become incorporated into oligomers. Therefore, on suitable membranes, oligomerization of daptomycin approaches or reaches completion.

Titration of excimer fluorescence with phosphatidylglycerol. In previous studies [6, 11, 14], phosphatidylglycerol was used in considerable molar excess over daptomycin, and therefore these experiments did not provide information on the stoichiometry of the interaction of daptomycin with PG. To examine this question, we used a mixture of DMPC and DMPG with an excess of dihexanoyl-PC. Mixtures of the latter with lipids of typical acyl chain length yield lipid bicelles, which are small, disk-like lipid aggregates that are suitable as membrane mimetics [15]. The advantage of bicelles over liposomes is twofold. Firstly, suspensions of bicelles are much less turbid. This allows measurements at higher concentrations of daptomycin and PG, which will help to drive the reaction between the two toward completion. Secondly, in a bicelle, both monolayers will be exposed to daptomycin. In contrast, with liposomes, only the outer monolayer may be accessible. Previous observation of membrane fusion induced by daptomycin in model liposomes [14] suggests that the membrane continuity may be disrupted, and both monolayers may be accessible to daptomycin in this system also. On the other hand, membrane fusion did not go to completion. In addition, no morphological changes to the cell membranes of daptomycin-treated bacteria were observed by electron microscopy [16], suggesting that the inner monolayer me not or only incompletely accessible to daptomycin. Use of bicelles avoids this uncertainty.

Figure 7 shows the titration of 100 μM perylene-daptomycin, in 20 mM dihexanoyl-PC and 1 mM calcium, with a mixture of dimyristoyl-PC and dimyristoyl-PG. Excimer formation is monitored as the ratio of fluorescence intensities at wavelengths 521 nm and 482 nm. It can be seen that the extent of excimer formation, and thus oligomerization, approaches saturation after addition of a stoichiometric amount of phosphatidylglycerol.

Discussion

In this study, we synthesized pyrene-daptomycin and perylene-daptomcyin and used the latter to characterize the oligomerization of daptomycin on membranes. The perylene moiety was introduced into daptomycin by substituting it for the normal decanoyl chain. Considering the substantial bulk that is added to the molecule by this modification, the fact that the antimicrobial activity is reduced no more than threefold is quite remarkable, as is the observation that the introduction of pyrene caused no measurable decrease in activity at all. These observations are, however, in line with a previous study on a variety of acyl analogues, which revealed only minor changes to the antibacterial activity, as long as the overall hydrophobic character of the acyl chain was preserved [9]. While the spectral overlap between pyrene and kynurenine prevented the observation of pyrene excimer fluorescence, excimers could be observed and used to characterize the oligomerization of daptomycin.

The perylene excimer fluorescence indicated the formation of daptomycin oligomers on both liposomes and bacterial cells. In liposomes, oligomerization correlates with the presence of phosphatidylglycerol. These findings are entirely consistent with those of a previous study that used fluorescence energy transfer [6]. Beyond this confirmation, the use of perylene-daptomcyin provides significant additional insights that could not be inferred from previous experiments.

The first novel observation is the formation of daptomycin oligomers on live bacterial cells. Secondly, using perylene-daptomcyin, the extent of oligomerization could be measured. This extent exceeds 90% on model membranes that contain 50% DMPG, and it is only slightly lower on bacterial membranes. This high extent of oligomerization supports the notion that it is the oligomer that causes the bactericidal effect.

Thirdly, the formation of perylene excimers shows that, in the oligomer, the N-terminal acyl chains interact directly with one another. Direct interaction of the daptomycin peptide moieties

within the oligomer was previously inferred from the self-quenching of NBD-labels that had been attached to their ornithine residues [6]. Taken together, these two points of mutual interaction suggest that neighboring oligomer subunits are aligned in a parallel fashion, or form an acute angle with one another.

The fourth novel finding reported here is that daptomycin oligomerization is driven by a stoichiometric interaction with phosphatidylglycerol. This result suggests that PG interacts with daptomycin directly and specifically, rather than indirectly through influencing bulk properties of the membrane such as charge or lateral phase segregation. Enhanced conversion of PG to lysyl-PG by bacterial cells has been reported as a mechanism of partial resistance to daptomycin [4, 5], which supports the notion that PG-mediated oligomerization is essential for the activity of daptomycin.

In summary, the current study provides significant new insights into the interaction of daptomycin with itself and with membrane lipids. At the same time, this study is the first one to use perylene excimer fluorescence for the characterization of peptide or protein oligomerization. The results provided here illustrate that perylene should be a valuable tool wherever the occurrence of intrinsic chromophores or fluorophores precludes the use of pyrene-labeled probes.

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References

[1] Tally, F.P., Zeckel, M., Wasilewski, M.M., Carini, C., Berman, C.L., Drusano, G.L. and Oleson, F.B. Daptomycin: a novel agent for Gram-positive infections, Expert Opin Investig Drugs *8* (1999) 1223-1238.

[2] Tally, F.P. and DeBruin, M.F. Development of daptomycin for gram-positive infections, J. Antimicrob. Chemother. *46* (2000) 523-526.

[3] Silverman, J.A., Perlmutter, N.G. and Shapiro, H.M. Correlation of daptomycin bactericidal activity and membrane depolarization in Staphylococcus aureus, Antimicrob. Agents Chemother. *47* (2003) 2538-2544.

[4] Hachmann, A., Angert, E.R. and Helmann, J.D. Genetic analysis of factors affecting susceptibility of Bacillus subtilis to daptomycin, Antimicrob. Agents Chemother. *53* (2009) 1598-1609.

[5] Hachmann, A., Sevim, E., Gaballa, A., Popham, D.L., Antelmann, H. and Helmann, J.D. Reduction in membrane phosphatidylglycerol content leads to daptomycin resistance in Bacillus subtilis, Antimicrob. Agents Chemother. *55* (2011) 4326-4337.

[6] Muraih, J.K., Pearson, A., Silverman, J. and Palmer, M. Oligomerization of daptomycin on membranes, Biochim. Biophys. Acta *1808* (2011) 1154-1160.

[7] Lakowicz, J. (1999). Principles of Fluorescence Spectroscopy, 2. Edition (Springer).

[8] Johnson, P. and Offen, H. Perylene excimer fluorescence in cyclohexane, Chemical Physics Letters *18* (1973) 258-260.

[9] Debono, M., Abbott, B.J., Molloy, R.M., Fukuda, D.S., Hunt, A.H., Daupert, V.M., Counter, F.T., Ott, J.L., Carrell, C.B. and Howard, L.C. Enzymatic and chemical modifications of lipopeptide antibiotic A21978C: the synthesis and evaluation of daptomycin (LY146032), J. Antibiot. *41* (1988) 1093-1105.

[10] Mayer, L.D., Hope, M.J. and Cullis, P.R. Vesicles of variable sizes produced by a rapid extrusion procedure, Biochim. Biophys. Acta *858* (1986) 161-168.

[11] Lakey, J.H. and Ptak, M. Fluorescence indicates a calcium-dependent interaction between the lipopeptide antibiotic LY146032 and phospholipid membranes, Biochemistry *27* (1988)

4639-4645.

[12] Epand, R.M., Rotem, S., Mor, A., Berno, B. and Epand, R.F. Bacterial membranes as predictors of antimicrobial potency, J. Am. Chem. Soc. *130* (2008) 14346-14352.

[13] López, C.S., Heras, H., Garda, H., Ruzal, S., Sánchez-Rivas, C. and Rivas, E. Biochemical and biophysical studies of Bacillus subtilis envelopes under hyperosmotic stress, Int. J. Food Microbiol. *55* (2000) 137-142.

[14] Jung, D., Powers, J.P., Straus, S.K. and Hancock, R.E.W. Lipid-specific binding of the calcium-dependent antibiotic daptomycin leads to changes in lipid polymorphism of model membranes., Chem. Phys. Lipids *154* (2008) 120-128.

[15] Glover, K.J., Whiles, J.A., Wu, G., Yu, N., Deems, R., Struppe, J.O., Stark, R.E., Komives, E.A. and Vold, R.R. Structural evaluation of phospholipid bicelles for solution-state studies of membrane-associated biomolecules, Biophys. J. *81* (2001) 2163-2171.

[16] Cotroneo, N., Harris, R., Perlmutter, N., Beveridge, T. and Silverman, J.A. Daptomycin exerts bactericidal activity without lysis of Staphylococcus aureus, Antimicrob. Agents Chemother. *52* (2008) 2223-2225.

Legends to Figures

Figure 1: Structure of daptomycin, and of the substituents pyrene- and perylene-butanoic acid that were substituted for the decanoyl residue of the native compound in this study.

Figure 2: Fluorescence emission spectra of pyrene-daptomycin in solution and on DMPC/DMPG liposomes, in the presence of 6 mM calcium. The excitation wavelength was 340 nm.

Figure 3: Fluorescence emission spectra of perylene-daptomycin, in solution (A), on DMPC liposomes (B), on DMPC/DMPG liposomes (C), and on bacterial cells (D). The emission spectra were measured with 2.5 μM perylene-daptomycin. In (A), the absorption spectra of perylene-daptomycin (PD) and of native daptomycin (ND) at 50 μM in solution are also shown. The absorption spectrum of native daptomycin on membranes is indistinguishable (not shown). In (D), the cells were incubated with perylene-daptomycin and calcium, then washed twice by centrifugation to remove unbound daptomycin and resuspended before acquisition of the spectrum.

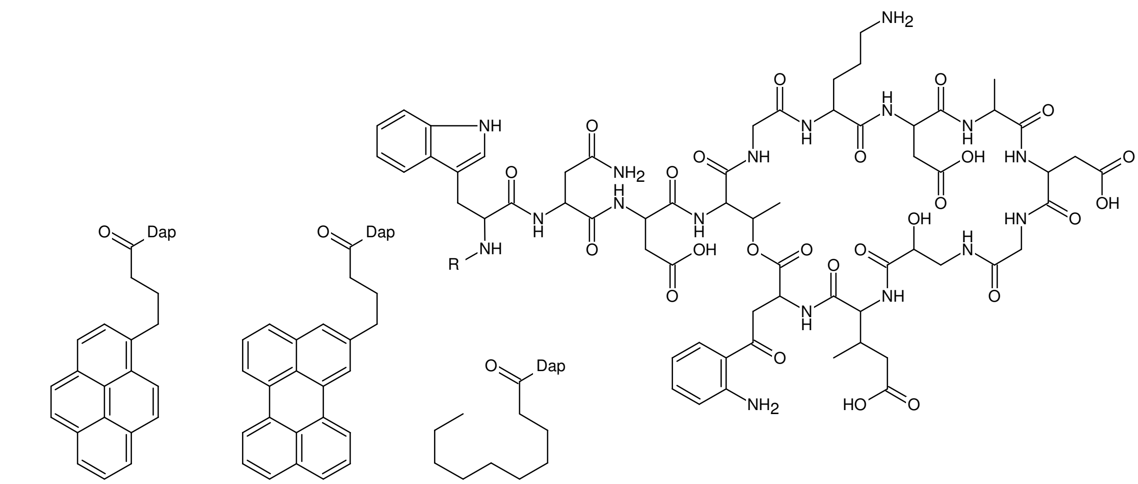
Figure 4: Time-resolved fluorescence measurements of perylene-daptomycin on DMPC (A) and DMPC/DMPG (B) membranes. Sample composition was as in Figure 3B and 3C, respectively. Excitation was with a 440 nm diode laser. Emission was measured using a 560±10 nm bandpass filter. The decays were fitted to a two-exponential model. Pre-exponentials (α) and lifetimes (τ) of the fitted components, as well as the residuals and χ^2 of the fits are given.

Figure 5: Effect of unlabeled daptomyin on the extent of excimer formation by perylene-daptomycin. Perylene-daptomycin (2.5 μM) was mixed with unlabeled daptomycin at the molar ratios indicated and incubated with DMPG/DMPC or DMPC liposomes with 6 and 200 mM of calcium, respectively. The change in fluorescence is represented the ratio of emission at 521 nm over that at 482 nm; a higher ratio indicates a greater extent of excimer formation.

Figure 6: Deconvoluted excimer and monomer spectra of perylene-daptomycin on DMPG/DMPC membranes. The steps involved in the deconvolution are described in the Methods section.

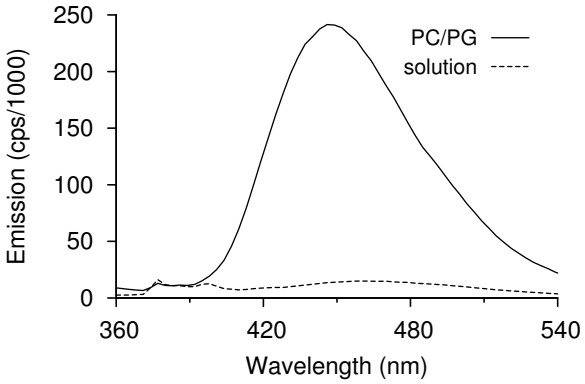
Figure 7: Titration of excimer formation with DMPG. Perylene-daptomycin was incubated with dihexanoyl-PC (20 mM) and calcium (1 mM). A suspension of DMPC/DMPG liposomes was added in a stepwise fashion, and the perylene fluorescence emission recorded after each addition. The increment in the ratio of the emissions at 521 nm and at 482 nm is plotted as a function of the molar ratio of PG to daptomycin. The curve represents a fit to the law of mass action, and the straight lines represent the tangents of the fit curve at zero and infinity, respectively.

Figure 1



R= pyrene-butanoate perylene-butanoate decanoate daptomycin

Figure 2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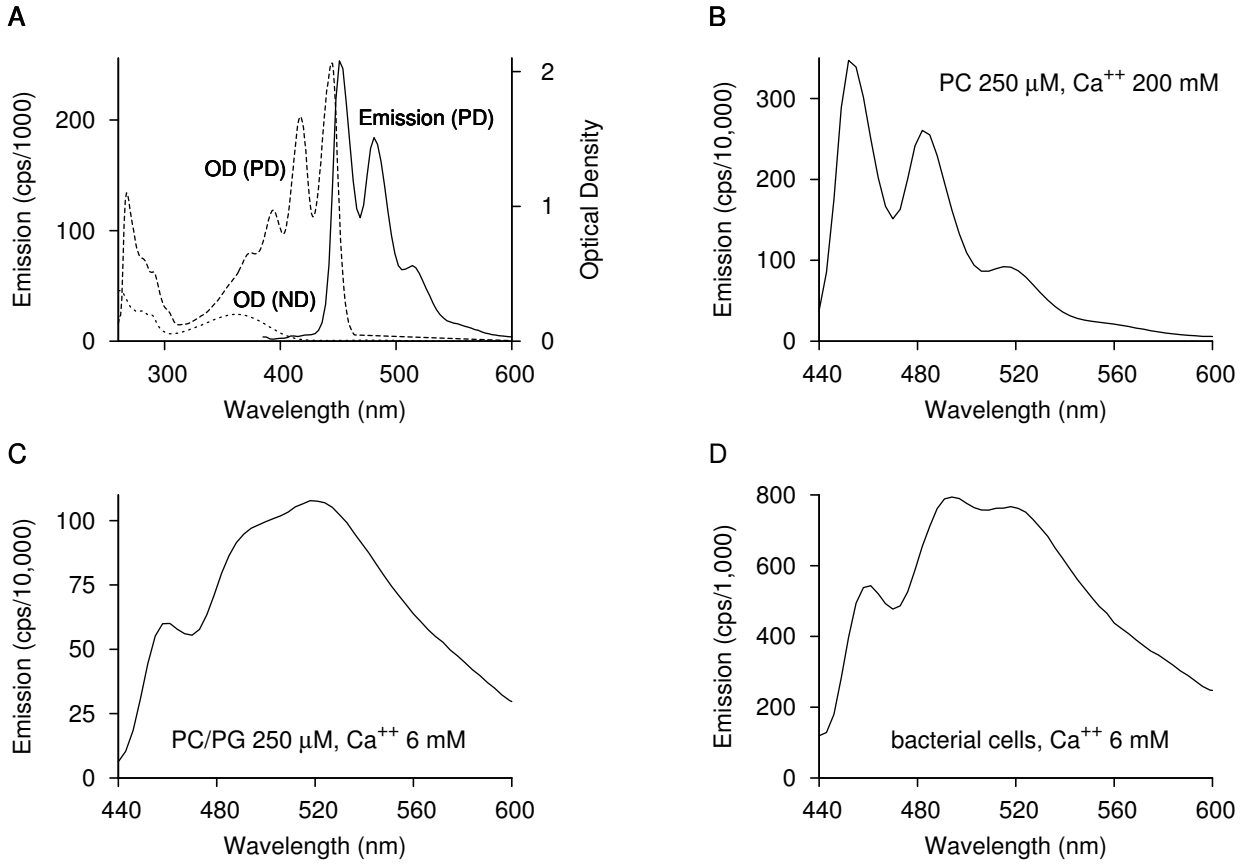
420
480
540
Wavelength (nm)
PC/PG
solution

Figure 3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Optical Density
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PC 250 µM, Ca++ 200 mM
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PC/PG 250 µM, Ca++ 6 mM
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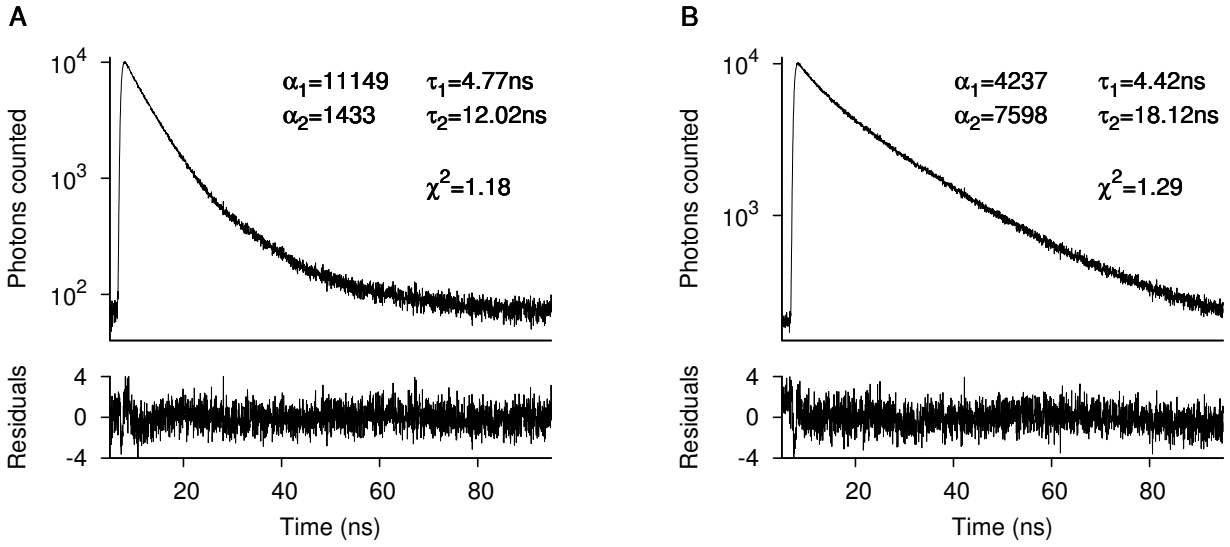
Figure 4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B
Photons counted
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α1=11149
τ1=4.77ns
α2=1433
τ2=12.02ns
χ2=1.18
α1=4237
τ1=4.42ns
α2=7598
τ2=18.12ns
χ2=1.29

Figure 5

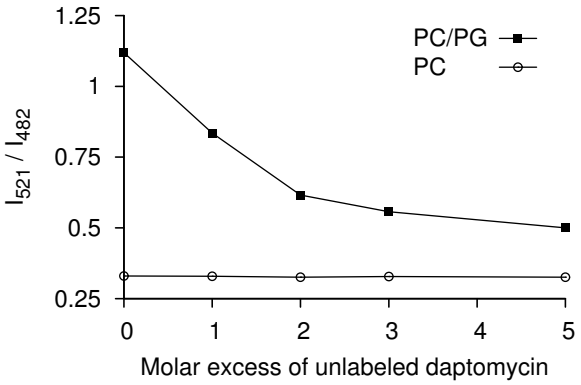

1.25
1
0.75
0.5
0.25
I_{521} / I_{482}
PC/PG
PC
0
1
2
3
4
5
Molar excess of unlabeled daptomycin

Figure 6

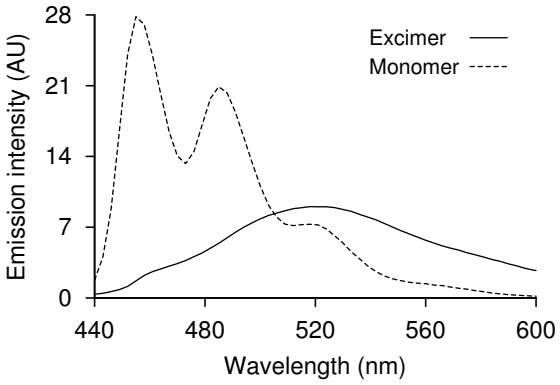
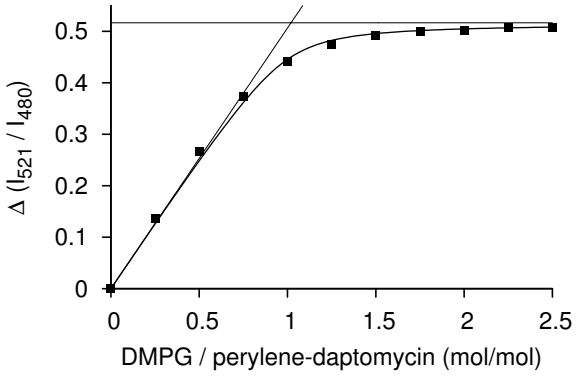
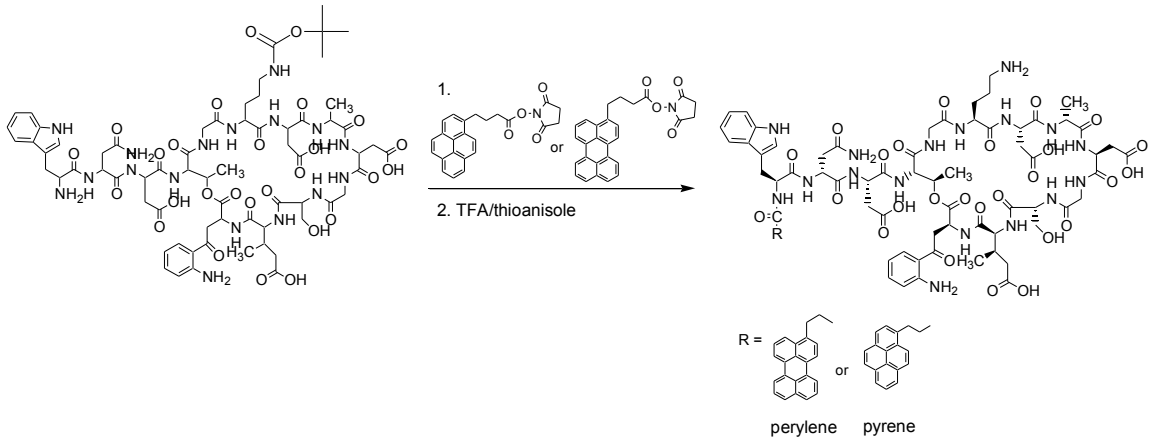

Emission intensity (AU)
28
21
14
7
0
440
480
520
560
600
Wavelength (nm)
Excimer
Monomer

Figure 7


0.5
0.4
0.3
0.2
0.1
0
Δ (I521 / I480)
0
0.5
1
1.5
2
2.5
DMPG / perylene-daptomycin (mol/mol)

General. DMF was distilled under reduced pressure from CaH. All reagents used in syntheses were obtained from Aldrich Chemical Co (Milwaukee, Wisc., USA) except for 1-pyrenebutanoic acid succinimidyl ester which was obtained from Anaspec Inc (Freemont, CA, USA). Deacylated daptomycin bearing a Boc protecting group on the ornithine residue was a gift from Cubist Inc (Boston, Mass., USA). Flash chromatography was performed using silica gel 60Å (234-400 mesh) obtained from Silicycle (Laval, Quebec, Canada). ^{1}H NMR was performed on a Bruker Avance 300. Chemical shifts (δ) are reported in ppm relative to the internal standard tetramethylsilane (TMS). Negative ion electrospray mass spectrometry ($^-$ESIMS) was performed on a Waters/Micromass QTOF Ultima Global mass spectrometer using 1:1 CH_3CN/H_2O (0.5% ammonium hydroxide) as solvent. Analytical and semi-preparative HPLC was performed on a Waters 600E system equipped with Waters 474 scanning fluorescence detector ($\lambda_{excitation}$ = 365 nm, $\lambda_{emission}$ = 460 nm). Semi-preparative HPLC was performed using a Higgins (Higgins Analytical Inc., Mountainview, CA. USA) Proto-200 C-18 semi-preparative (20 mm x 250 mm) column with a flow rate of 6 mL/min. Analytical HPLC was performed using a Phenomenex (Torrance, CA, USA) Jupiter Proteo C-18 analytical (4.6 x 250 mm) column with a flow rate of 1 mL/min.

Perylene- and pyrene-daptomycin was prepared according to Scheme 1. Details of the procedure are given in the Methods section of the main text.



Scheme 1. Synthesis of perylene- and pyrene-daptomycin.

4-(3-Perylenyl)butanoic acid succinimidyl ester. This compound was prepared using a procedure based upon that reported by Oskolkova et al. *(1)* 4-(3-Perylenyl)butanoic acid (0.030g, 0.089 mmol) was added to a solution of *N*-hydroxysuccinimide in dichloromethane (2 mL) and cooled to 0 ^{o}C (ice bath). To this was added *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC, 0.017 g, 0.098 mmol) and a catalytic amount of dimethylaminopyridine (DMAP). The solution was stirred at 0 ^{o}C for 1h. The ice bath was removed and the solution stirred for an additional 24 h. The solution was diluted with dichloromethane (10 mL) and washed with sat. brine (3 x 10 mL). The organic layer was dried (Na_2SO_4) and concentrated by rotary evaporation. Flash chromatography (40% ethyl acetate, 60% hexane, $R_f = 0.3$) of the residue gave pure 4-(3-perylenyl)butanoic acid succinimidyl ester in a 90% yield (0.030 g) as a yellow amorphous solid. ^{1}H NMR ($CDCl_3$): δ 2.22 (quint, 2H, J = 7.9 Hz), 2.73 (t, 2H, J = 6.9 Hz), 2.87 (s, 4H), 3.17 (t, 2H, J = 7.4 Hz), 7.37 (d, 1H, J = 7.4 Hz), 7.43-7.59 (m, 3H), 7.66 (d, 1H, J = 3.7 Hz), 7.68 (d, 1H, J = 3.7 Hz), 7.89 (d, 1H, J = 8.5 Hz), 8.10-8.25 (m, 4H).

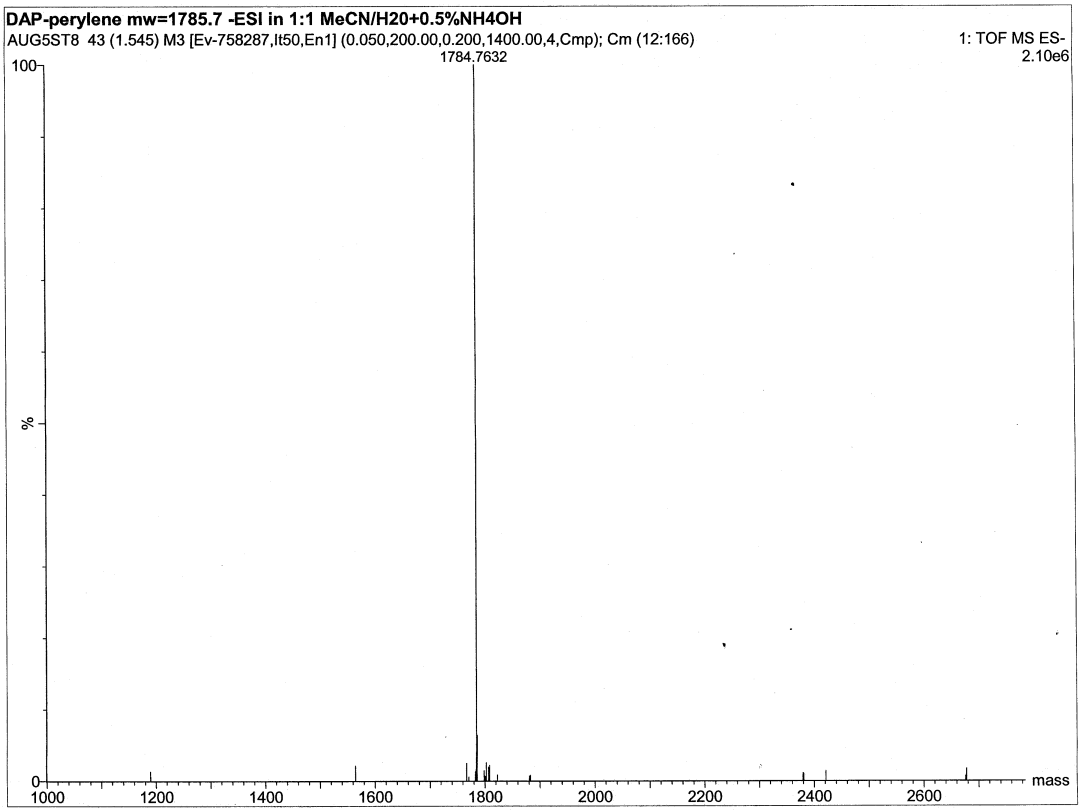


Figure S1. $^{-}$ESIMS of dap-perylene. The peak with *m/z* 1784.76 corresponds to the $(M-H)^{-1}$ ion.

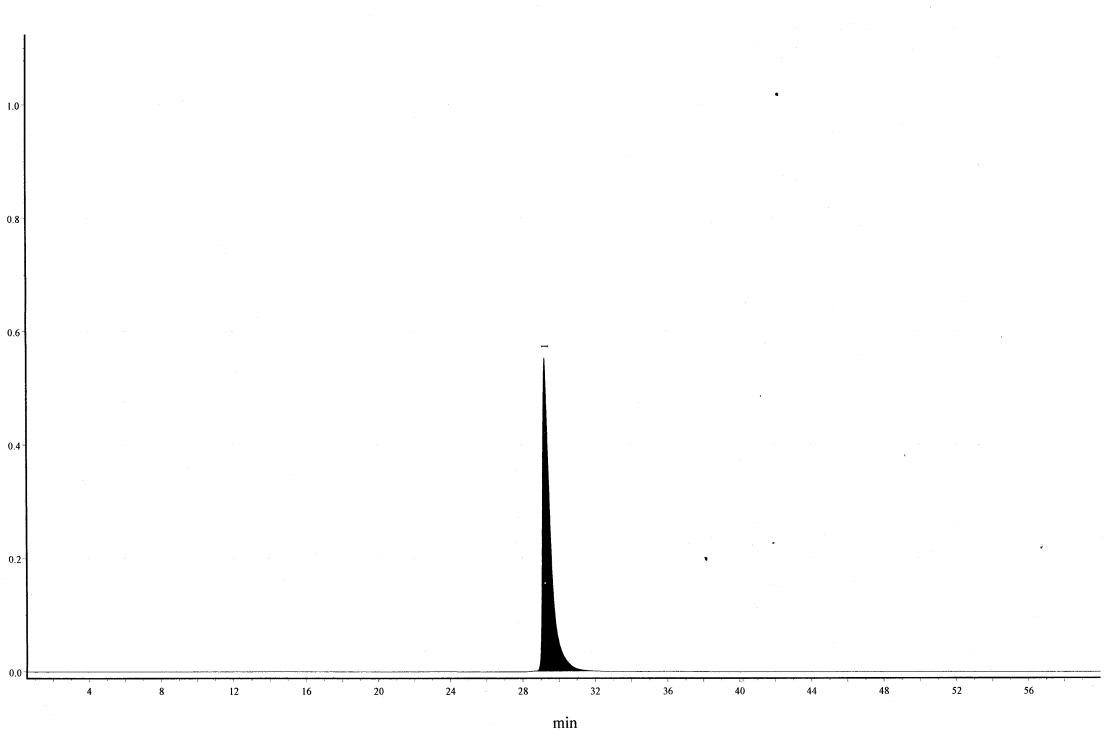


Figure S2. Analytical HPLC chromatogram of dap-perylene (t_r = 29.5 min) after HPLC purification. Analytical HPLC was performed using a Phenomenex Jupiter Proteo C-18 analytical column (250 x 4.6 mm), a linear gradient starting with 95% water (0.1% TFA)-5% acetonitrile to 100 % acetonitrile over 50 minutes with a flow rate of 1 mL/min ($\lambda_{excitation}$ = 365 nm, $\lambda_{emission}$ = 460 nm).

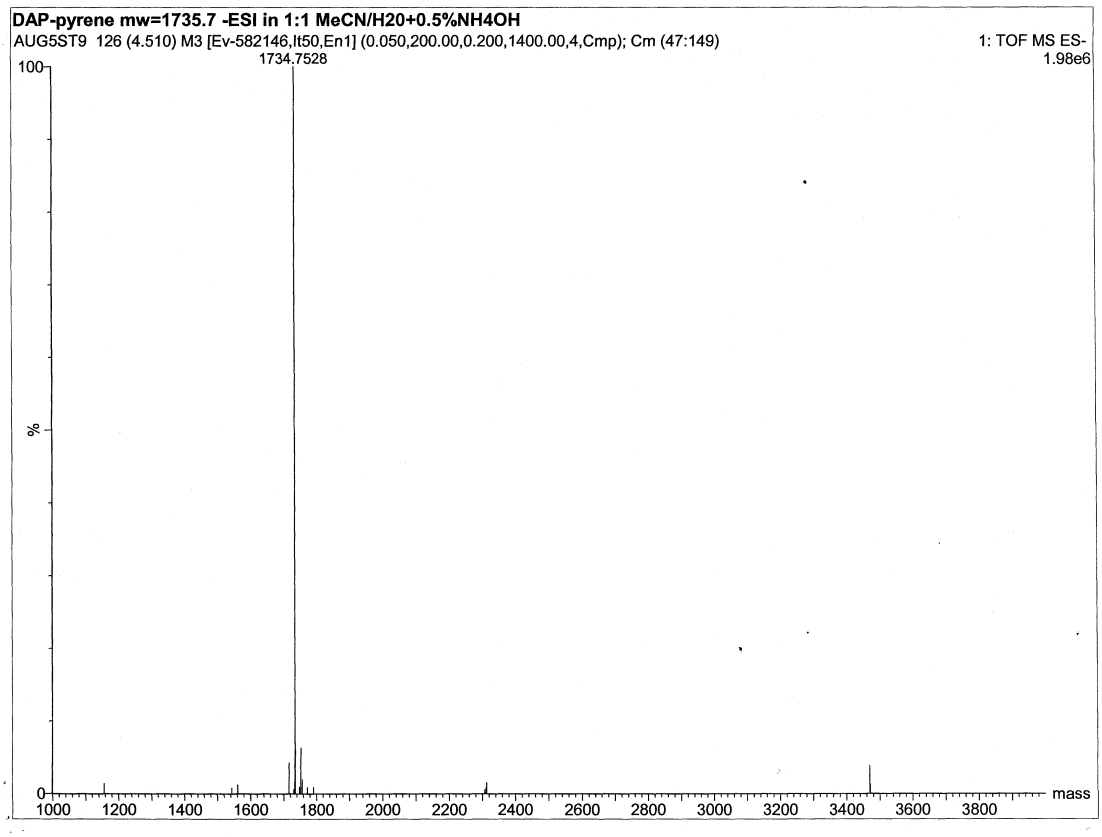


Figure S3. $^{-}$ESIMS of dap-pyrene. The peak with *m/z* 1734.75 corresponds to the $(M-H)^{-}$ [1] ion.

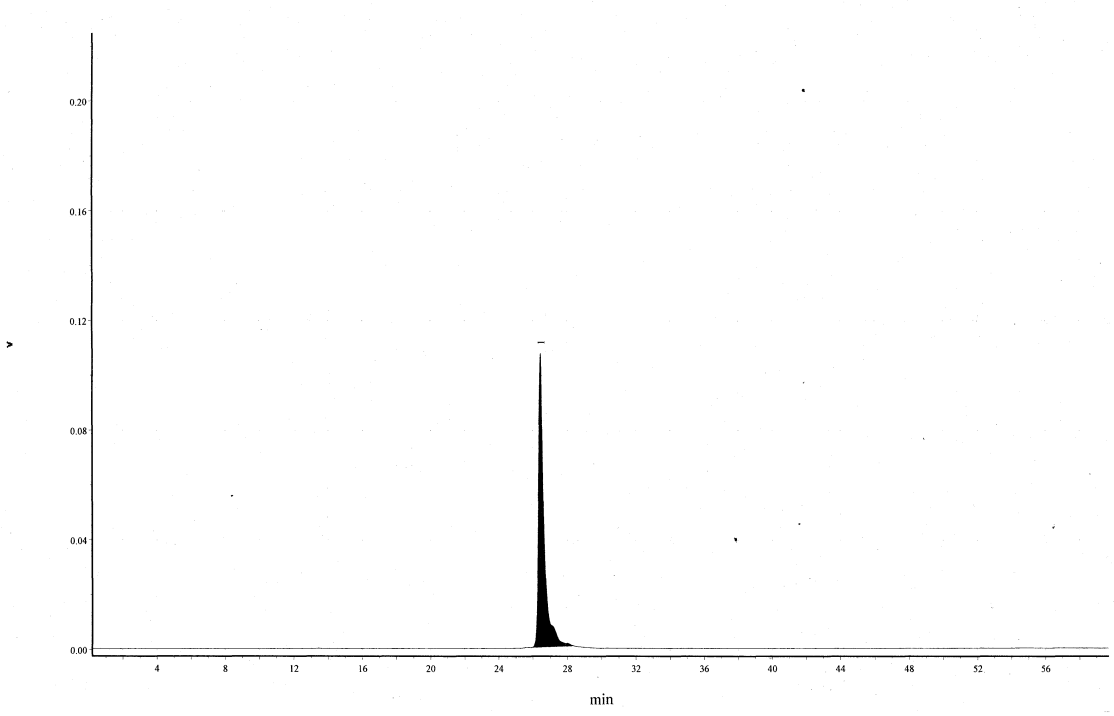


Figure S4. Analytical HPLC chromatogram of dap-pyrene (t_r = 26.4 min) after HPLC purification. Analytical HPLC was performed using a Phenomenex Jupiter Proteo C-18 analytical column (250 x 4.6 mm), a linear gradient starting with 95% water (0.1% TFA)-5% acetonitrile to 100 % acetonitrile over 50 minutes with a flow rate of 1 mL/min ($\lambda_{excitation}$ = 365 nm, $\lambda_{emission}$ = 460 nm).

References

1. Oskolkova, O.V., Saf, R., Zenzmaier, E. and Hermetter, A. (2003) Fluorescent organophosphonates as inhibitors of microbial lipases, *Chem. Phys. Lipids 125*, 103-114.