A Diazirine-Modified Membrane Lipid to Study Peptide/Lipid Interactions – Chances and Challenges

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1. Synthesis

1.1. DiazPC (P12DiazSPC)

Reactions and conditions:
i) pyridinium dichromate, CHCl₃, DMF, 0 °C; ii) NH₃, hydroxylamine-O-sulfonic acid, MeOH, 0 °C; iii) I₂, MeOH, red light; iv) lyso-PPC, CH₂Cl₂, MNBA, DMAP, r.t.

Scheme S1: Reaction scheme for the preparation of DiazPC (P12DiazSPC).

1.1.1. Synthesis of 12-oxo-octadecanoic acid (2)

12-Hydroxyoctadecanoic acid (25 g; 83.2 mmol) was dissolved in dry CHCl₃ (300 mL) and the solution was cooled to 0 °C on an ice bath. A solution of one equivalent pyridinium dichromate (31.3 g) in DMF (75 mL) was added dropwise. Afterwards, the ice bath was removed, and the mixture was stirred for 24 h at room temperature. A diluted solution of sulfuric acid (pH 2, 400 mL) was added, the organic layer was separated, and the water phase was extracted several times with CHCl₃. The combined organic phases were dried over Na₂SO₄ and purified by column chromatography on silica using a CHCl₃/Et₂O as solvent and the gradient technique. To remove residues of chromium salts, the evaporated product was dissolved in Et₂O and washed several times with water (pH 2) to get a clear organic phase. The organic phase was evaporated yielding 12-oxo-octadecanoic acid (2) as a white solid (10.6 g, 35.5 mmol, 43%). \( R_f \) (CHCl₃/Et₂O 8/2) = 0.34; \( ^1H\)-NMR: (400 MHz, CDCl₃, 27 °C) \( \delta = 0.88 \) (t, \( ^3J = 6.8 \text{ Hz}, 3\text{H}, \text{–CH₃} \)), 1.22–1.38 (m, 18H, CH₂), 1.52–1.59 (m, 4H, CH₂CH₂COCH₂CH₂), 1.61–1.67 (m, 2H, CH₂CH₂COOH), 2.33–2.47 (m, 6H, CH₂CO); HRMS (m/z): calc. for C₁₈H₃₃O₃ 297.2435 [M − H]⁻, found: 297.2405.

1.1.2. Synthesis of 12,12-diazi-octadecanoic acid (4)

Compound 4 was synthesized according to literature.[1] To form the diaziridine compound 3, keto acid 2 (0.54 g, 1.81 mmol) was dissolved in dry MeOH (30 mL) under argon atmosphere and was further cooled to 0 °C on an ice bath. Dry ammonia gas was then introduced through a gas inlet pipe into the solution for 2 h. Afterwards, hydroxylamine-O-sulfonic acid (2 equiv., 0.45 g, 3.98 mmol) dissolved in MeOH (20 mL) was added dropwise. After stirring for 30 min at 0 °C and further 4 h at room temperature, the suspension was filtered. The precipitate was washed with MeOH (20 mL). Triethylamine (TEA, 4 mL) was added to the combined MeOH phases. Afterwards, the solution of 3 was evaporated to a final volume of about 5 mL and water (100 mL) was added. Afterwards, the aqueous solution was extracted several times with ethyl acetate. The combined organic layers were dried over Na₂SO₄ and the crude product was purified by column chromatography on silica (and protection
from UV light) using a mixture of heptane/ethyl acetate/acetic acid (95/4/1, v/v/v) as solvent yielding 12,12-diazi-octadecanoic acid (4) as a white solid (0.11 g, 0.35 mmol, 20%). \( R_f \) (heptane/ether/acetic acid 5/5/0.1) = 0.5; HRMS (m/z): calc. for C\(_{18}\)H\(_{33}\)N\(_2\)O\(_2\) 309.2548 [M - H]\(^-\), found: 309.2527.

1.1.3. Synthesis of 1-palmitoyl-2-(12,12-diazi-octadecanoyl)-sn-glycero-3-phosphocholine (P12DiazSPC, DiazPC)

According to the literature, \(^2\) compound 4 (0.11 g, 0.354 mmol) and 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (lyso-PPC, 0.37 g, 0.75 mmol) were dissolved in dry CH\(_2\)Cl\(_2\) (15 mL) under argon atmosphere. Then, two equivalents of 2-methyl-6-nitrobenzoic anhydride (MNBA, 0.24 g, 0.7 mmol) and four equivalents of 4-(dimethylamino)pyridine (DMAP, 0.17 g, 1.4 mmol) were added and the mixture was stirred at room temperature. After 1 h, no starting material (compound 4) was detected via thin layer chromatography (TLC) and MeOH (5 mL) was added to stop the reaction. The solvent was evaporated, and the crude product was purified by column chromatography on silica starting with a mixture of CHCl\(_3\)/MeOH 85/15 as solvent. The desired product was eluted using a final solvent mixture of CHCl\(_3\)/MeOH/H\(_2\)O 65/35/2.5 (v/v/v), evaporated, and dried via lyophilization—yielding the diazirine-modified phospholipids DiazPC as a white solid (0.13 g, 0.17 mmol, 49%). \( R_f \) (CHCl\(_3\)/MeOH/NH\(_3\) 65/35/5) = 0.38; \(^1\)H-NMR: (500 MHz, CDCl\(_3\), 27 °C) \( \delta = 0.88 \) (td, \( J = 6.9, 5.3 \) Hz, 6H, 2CH\(_2\)C\(_3\)H\(_3\)), 0.98–1.12 (m, 4H, C\(_\text{H}_2\)C(N=N)C\(_\text{H}_2\)), 1.14–1.51 (m, 50H, C\(_\text{H}_2\)), 2.23–2.34 (m, 4H, C(O)OC\(_\text{H}_2\)CH\(_2\)), 3.41 (s, 9H, N(C\(_\text{H}_3\))\(_3\)), 3.86–4.48 (m, 8H, OC\(_\text{H}_2\)C\(_\text{H}_2\)N and OC\(_\text{H}_2\)CH\(_2\)O) (Figure S2); HRMS (m/z): calc. for C\(_{42}\)H\(_{82}\)N\(_3\)O\(_8\)PNa 810.5732 [M + Na]\(^+\), found: 810.576; calc. 1204.3652 [3M + 2Na]\(^2+\), found: 1204.369; calc. 1598.1571 [2M + Na]\(^+\), found: 1598.162 (Figure S1).

1.2. Model Peptide LAVA20

In our previous study, we used the \( \alpha \)-helical model peptide named KLAW23\(^{[2a]} \) (sequence: Ac-GKK(LA)\(_8\)LWWA-NH\(_2\)), which is a modified version of the well-known transmembrane model peptides WALP and KALP, respectively, that have been extensively studied by Killian and co-workers.\(^{[3]} \) However, it was found in preliminary studies that the exact position of cross-links within the transmembrane helix of KLAW23 was difficult to determine due to the repetitive leucine–alanine (LA) sequence. We therefore substituted some of the leucine with valine (V). In addition, we reduced the overall length of the model peptide from 23 to 20 amino acids so that the hydrophobic helix would fit well with a DOPC membrane, which has a hydrocarbon core thickness of \( d_C = 2.71 \) nm in the fluid state.\(^{[4]} \) Lastly, we added one additional lysine (K) at the C terminus to obtain singly charged y-type ions for MS.

According to a procedure described elsewhere,\(^{[2a]} \) the peptide Ac-GKKLAVAVALAVALALWWAK-NH\(_2\) (C\(_{104}\)H\(_{171}\)N\(_{26}\)O\(_{21}\), monoisotopic mass: \( m/z \) 2120.31066; referred to as LAVA20) was synthesized using fast Fmoc-SPPS\(^{[5]} \) on a Tetras Peptide Synthesizer (Advanced Chemtec, Louisville, KY, USA) (at 60 µmol scale) using a standard protocol. Fmoc-amino acids (Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Val-OH, Fmoc-Trp(Boc)-OH, Fmoc-Lys(Boc)-OH, and Ac-Gly-OH; \( c = 0.2 \) M in DMF) were used at tenfold excess relative to ChemMatrix® H-Rink amide resin.\(^{[6]} \) Coupling was performed using amino acid/N,N,N′,N′-tetramethyl-O-(benzotriazol-1-yl)uronium tetrafluoroborate (TBTU)/4-methylmorpholine (NMM) (1/1/2, \( n/n/n \)) in DMF for 5 min. Fmoc-deprotection was carried out using piperidine/1,8-diazabicyclo[5.4.0]undec-7-ene (DBU)/DMF (20/2/78, \( v/v/v \)) for 2× 1 min. Thorough DMF washes were performed after each coupling and deprotection step. The final cleavage and the deprotection of the peptide were performed by means of TFA/water/triisopropylsilane (TIPS) (91:6:3, \( v/v/v \)). The crude peptide was precipitated with cold diethyl ether, centrifuged, and the residue was purified by semipreparative HPLC (Merck Hitachi, Phenomenex, Luna C18(2) column) using water/acetonitrile as eluent. The purity (99%) and identity of the purified peptide was confirmed by HPLC (Figure S3) and MALDI-TOF-MS (Figure S4 and Figure S5), respectively.
2. Analytical data

2.1. DiazPC (P12DiazSPC)

Figure S1. HRMS of DiazPC.
Figure S2. $^1$H-NMR of DiazPC.
2.2. LAVA20

Figure S3. HPLC of model peptide LAVA20.
Figure S4. MALDI-TOF-MS of model peptide LAVA20.
Figure S5. MALDI-TOF-MS of model peptide LAV20.
3. Physicochemical Investigations

3.1. Chemicals

1-Dipalmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids (Alabaster, USA). 12-Hydroxystearoic acid, other chemicals for the synthesis, and buffer salts were purchased from Sigma Aldrich Co. (Steinheim, Germany).

3.2. Physicochemical Methods

3.2.1. Sample Preparation

The appropriate amount of pure DiazPC was suspended in H2O (Milli-Q Millipore water with a specific resistance $\rho = 18.2$ M$\Omega$ cm). Homogeneous suspensions were obtained by several cycles of heating to 90 °C and vortexing. Binary lipid mixtures were prepared from lipid stock solutions in CHCl3/MeOH (2/1, v/v) as solvent by mixing appropriate volumes of the stock solutions. Afterwards, the organic solvent was removed in a stream of nitrogen and the resulting lipid films were kept in an evacuated flask for at least 12 h to remove residual traces of solvent. The suspensions were then prepared by adding a certain volume of Milli-Q water or phosphate buffer (10 mM, pH = 7.4–7.5) to obtain a total lipid concentration of $c = 3$ mM. The samples were vigorously vortexed and heated to 90 °C several times to obtain a homogeneous suspension. Liposomes were prepared by extrusion (31 times) of the lipid suspension through a polycarbonate membrane (100 nm) at a temperature approximately 10 K above the transition temperature observed in the DSC experiments. Liposomes containing 2 mol% LAVA20 were prepared by five freeze/thaw cycles (using liquid nitrogen and a water bath of 60 °C) followed by extrusion through a polycarbonate membrane (100 nm).

An important note: the preparation of the samples containing DiazPC and the experiments have to take place under light exclusion (using red light only!) to prevent the premature and uncontrolled reaction of the diazirine moiety.

3.2.2. UV Irradiation

The samples were placed in 1.5mL-Protein LoBind-Tubes (Eppendorf, Germany) and irradiated using the LED lamp Aicure UJ30 spot light type (Panasonic Electric Works Europe AG, Holzkirchen, Germany) working at $\lambda = 365$ nm and a maximal intensity of irradiation of 1.0–1.5 W cm². To investigate the optimal duration and intensity of UV irradiation, the samples were irradiated at an intensity of 10% and a duration of 2 s (UV1) or 22 s (UV2); then the intensity was increased to 100% and the samples were further irradiated for 0.2 s (UV3) or 2.2 s (UV4). For all other studies, irradiation was performed at an intensity of 100% for 5 s.

3.2.3. Differential Scanning Calorimetry (DSC)

DSC measurements were performed using a MicroCal VP-DSC differential scanning calorimeter (MicroCal Inc. Northampton, MA, USA). Before the measurements, the sample suspension and the buffer reference were degassed under vacuum while stirring. A heating rate of 60 K h⁻¹ was used, and the measurements were performed in the temperature interval from 5 to 75 °C. To check the reproducibility, three consecutive scans were recorded for each sample. The water/water and buffer/buffer baseline, respectively, was subtracted from the thermogram of the sample, and the DSC scans were evaluated using MicroCal Origin 8.0 software.

3.2.4. Transmission Electron Microscopy (TEM)

According to procedures described previously,[2a, 7] samples for TEM were prepared by spreading 5 µL of the lipid suspension ($c = 60$ µM) onto a copper grid coated with a Formvar film. After 1 min, excess liquid was blotted off with filter paper and 5 µL of 1% aqueous uranyl acetate solution were placed onto the grid, drained off after 1 min, and the samples were dried at room temperature for at least 24 h. All specimens were examined with an EM 900 transmission electron microscope (Carl Zeiss Microscopy, Oberkochen, Germany) and micrographs were recorded with an SM-1k-120 slow-scan charge-coupled device (slow-scan CCD) camera (TRS, Moorenweis, Germany).
3.2.5. Dynamic Light Scattering (DLS)

DLS experiments were carried out with an Litesizer 500 (Anton Paar GmbH, Graz, Austria). A 3-mW-laser with a wavelength $\lambda = 633$ nm and a scattering angle of $173^\circ$ was used. All samples ($c = 1.5$ mM) were filled into cuvettes (path length 10 mm). Before starting the measurement, each sample was equilibrated for at least 2 min at 20 °C. Three individual measurements were performed for each sample to test the reproducibility with one measurement consisting of 60 runs of 10 s each. The experimental data were analyzed with the aid of the Kallipoe software (Anton Paar GmbH, Graz, Austria).

3.2.6. Fourier-Transform Infrared Spectroscopy (FTIR)

Infrared spectra were collected on a Bruker Vector 22 Fourier transform spectrometer with DTGS detector operating at 2 cm$^{-1}$ resolution. The sample suspension ($c = 100$ mg mL$^{-1}$ in water) was placed between two CaF$_2$ windows, separated by a 6 µm spacer. IR spectra were recorded in 1-K-steps in the temperature range from 5 to 43 °C. The temperature was adjusted with a Haake F6 thermostat (C25, Thermo Electron Corporation, Karlsruhe, Germany) and controlled with Delphi-based home-written software. After an equilibration time of 8 min, 128 scans were recorded and accumulated. The corresponding spectra of the solvent (Milli-Q water) were subtracted from the sample spectra using the OPUS software supplied by Bruker.

3.2.7. Attenuated Total Reflection Fourier-Transform Infrared (ATR-FTIR) spectroscopy

According to a procedure described previously,[2a] lipid suspensions ($c = 3$ mM in PBS, pH 7.4) were analyzed using a Bruker BioATR2 unit with a Bruker Tensor27 FTIR spectrometer (Bruker Optics GmbH, Ettlingen, Germany). Single-channel IR spectra were recorded between 5,000 and 900 cm$^{-1}$ using unpolarized IR light with a resolution of 2 cm$^{-1}$. The error of the obtained wavenumber is ± 0.5 cm$^{-1}$. The temperature was adjusted with a Haake Pheonix II thermostat (C25P, Thermo Electron Corporation, Karlsruhe, Germany) and controlled with a Delphi-based home-written software. After an equilibration time of 8 min, 64 scans were recorded and accumulated at each temperature. Each sample was measured four times to check reproducibility. As reference, single-channel IR spectra (64 scans), taken from pure water at the appropriate temperature, were used and subtracted from the corresponding sample spectra using the Bruker OPUS software.

3.2.8. Small Angle X-ray Scattering (SAXS)

According to a procedure described previously,[8] SAXS measurements were performed with a laboratory SAXS instrument (Nanostar, Bruker AXS GmbH, Karlsruhe, Germany). The instrument includes an IµS micro-focus X-ray source with a power of 30 W using the wavelength of the Cu Kα line. As the detector, a VÅNTEC-2000 detector (14 × 14 cm$^2$ and 2048 × 2048 pixel) was used. The sample to detector distance was 108.3 cm and the accessible q range was from 0.01 to 0.23 Å$^{-1}$. Samples were filled into glass capillaries of 2 mm diameter with temperature control ($\Delta T = \pm 0.1$ K). The raw scattering data were corrected for the background from the solvent measured in a capillary with the same diameter and then converted to absolute units using the scattering of pure water measured at 20 °C (program SuperSAXS, Prof. C. L. P. Oliveira and Prof. J. S. Pedersen).

3.2.9. UV/Vis-Spectroscopy and Circular Dichroism (CD) Spectroscopy

The CD measurements were performed using a Jasco J-810 spectrometer (Jasco GmbH, Pfungstadt, Germany). DOPC liposomes (with and without the model peptide LAVA20) were prepared as described above ($C_{lipid} = 1.15$ mg mL$^{-1}$) using phosphate buffer (10 mM, pH 7.4). Since the diazirine group (DiazPC) absorb in the measured spectral range and would therefore interfere with the CD measurement, the addition of DiazPC was omitted. The lipid sample (200 µL) was filled into a cuvette with a pathlength of 0.1 mm. The spectra were recorded in a spectral range from 190 to 250 nm at a temperature of 20 °C using a scan rate of 50 nm min$^{-1}$. For each sample, 64 scans were recorded and accumulated to increase the signal-to-noise ratio.

For the determination of the peptide’s secondary structure fractions using the program CDNN it is necessary to determine an exact peptide concentration. Therefore, the absorbance of tryptophan at 280 nm was used. DOPC liposomes (with and without LAVA20) were measured twice each in a quartz glass cuvette with a path length of $d = 1$ mm. The light scattering signal of the pure DOPC liposomes was then subtracted from the sample.
The absorbance at a wavelength of 280 nm was collected from the corrected spectrum and the concentration \(c_{\text{Peptide}}\) was determined using Lambeert-Beer's law:

\[ A_{280} = \varepsilon_{280} \cdot c_{\text{Peptide}} \cdot d. \]  

(Eq 1)

The molar absorption coefficient (\(\varepsilon_{280}\)) depends on the number of Trp (W), Tyr (Y), and Cys (C) in the peptide/protein and can be estimated using the following equation:

\[ \varepsilon_{280} = \#_{\text{Trp}} \cdot 5600 \text{ M}^{-1}\text{cm}^{-1} + \#_{\text{Tyr}} \cdot 1490 \text{ M}^{-1}\text{cm}^{-1} + \#_{\text{Cys}} \cdot 125 \text{ M}^{-1}\text{cm}^{-1}. \]  

(Eq 2)

From this, we could calculate the concentration of LAVA20 to be \(c_{\text{LAVA20}} = 0.38 \pm 0.01 \text{ mM}\) corresponding to \(0.81 \pm 0.02 \text{ mg mL}^{-1}\).

3.2.10. Liquid chromatography mass spectrometry (LC–MS/MS)

LC–MS/MS analysis of DiazPC was performed on an Agilent 1200 HPLC System (Agilent Technologies, Santa Clara, CA, USA) equipped with an autosampler using the control software HyStar (Version:3.2, Bruker, Bremen, Germany) was used. Separation was performed at a column temperature of 50 °C with a Jupiter C4 column (Phenomenex, 150 × 2 mm, 5 μm, 300 Å) using a 45-minute gradient, in which the proportion of eluent B was increased linearly from an initial 10% to finally 90%. (Solvent A is 60 eq. water, 40 eq. acetonitrile, and 0.1 eq. formic acid; solvent B includes 90 eq. isopropyl alcohol, 10 eq. acetonitrile and 0.1 eq. formic acid.) A volume of 10 μL of the diluted sample solution (\(c_{\text{Lipid}} = 1 \text{ mM}\)) was injected.

The LC system was directly coupled to an Orbitrap Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with electrospray ionization (ESI) source. Data were generated via an automated MS/MS mode in which the ten most abundant signals of precursor ions were selected and fragmented. The normalized collision energy was 29% ± 3% for all measurements. MS analysis was performed at a resolution of \(R = 140,000\) at \(m/z\) 200. MS/MS analysis (\(R = 17,500\) at \(m/z\) 200) was performed within 5 s starting with the most abundant signal in the mass spectrum. The target volume was set to 200,000 and the maximum accumulation time to 250 ms. Dynamic exclusion of precursor ions was disabled. Xcalibur 4.1 software (Thermo Fisher Scientific, Bremen, Germany) was used to control the mass spectrometer and evaluate the mass spectra.
4. Results and Discussion

4.1. Aggregation Behaviour of DiazPC in Aqueous Dispersion

Figure S6. TEM image of an aqueous DiazPC suspension ($c = 0.05$ mg mL$^{-1}$). The sample was prepared at 20 °C and stained with uranyl acetate.

Figure S7. Wavenumber (heating, red; cooling, blue) of different vibrational band as a function of temperature for aqueous suspensions of DiazPC ($c = 100$ mg mL$^{-1}$ in water): (A) antisymmetric methylene stretching vibration and (B) methylene scissoring vibration.
**SUPPORTING INFORMATION**

**Table S1.** X-ray scattering data of DiazPC (c = 40 mg mL⁻¹) at different temperatures (as indicated).

| Sample  | T / °C | SAXS |  |
|---------|--------|------|---|
|         |        | q / nm⁻¹ | d / nm | FWHM / nm⁻¹ | ξ / nm     |
| DiazPC  | 5      | q₁ = 0.94  | 6.7 ± 0.1 | 0.08           | 70.7       |
|         |        | q₂ = 1.86  |          |               | (11 bilayers) |
|         | 30     | q₁ = 0.95  | 6.6 ± 0.1 | 0.08           | 70.7       |
|         |        | q₂ = 1.89  |          |               | (11 bilayers) |
|         | 30     | q = 1.51*  | 4.2 ± 0.1 | 0.43           |            |

d, repeat distance (i.e., thickness of bilayer + interlamellar water layer); FWHM, full-width at half-maximum; ξ = average size of stacked lamellar aggregates obtained from FWHM via Scherer equation[^10] and average number of bilayers.

[^10]: Very broad reflex of low intensity between both lamellar reflections (Q₁ and Q₂)

4.2. Degradation of DiazPC

The photoreaction of diazirines has been extensively described in the literature.[¹¹b,¹¹c] UV irradiation at λ = 365 nm generates reactive carbene intermediates after the loss of nitrogen. This carbene will then react non-specifically with various amino acid residues of membrane peptides/proteins or with adjacent alkyl chains of other phospholipids. However, carbenes additionally show several rearrangement reactions.[¹¹a] Thus, various unsaturated fatty acids, as well as cyclopropane[^12] and methylene-modified acyl chains can be formed by hydrogen shift (Scheme S2)—with the latter two occurring far less frequently. The formation of mono-unsaturated C₁₈ acids (C₁₈:₁,₁² and C₁₈:₁,₁¹—vaccenic acid) as degradation product of DiazPC is problematic in our case, since they have the same mass as oleic acid. Therefore, POPC cannot act as a mixing partner of DiazPC, since POPC has the identical mass as the degradation product of DiazPC, and DOPC was used instead.

**Scheme S2:** Reactions of DiazPC after UV Irradiation at 365 nm[^a]

[^a]: Abbreviations: P-C₁₈:₁,₁²-PC, 1-palmitoyl-2-(octadec-12-enoyl)-sn-glycero-3-phosphocholine; P-C₁₈:₁,₁¹-PC, 1-palmitoyl-2-(octadec-11-enoyl)-sn-glycero-3-phosphocholine; P-C₁₈cp,₁¹-PC, 1-palmitoyl-2-[(2-pentylcyclopropane)decanoyl]-sn-glycero-3-phosphocholine; P-C₁₇methylene,₁¹-PC, 1-palmitoyl-2(11-methyleneheptadecanoyl)-sn-glycero-3-phosphocholine.
**4.3. Mass Spectrometry**

**Table S2.** Results from MS of DiazPC/DOPC (1/2) liposomes including 2 mol% LAVA20 after UV irradiation.

| Ion | Charge | m/z     | t<sub>r</sub> / min |
|-----|--------|---------|---------------------|
| LAVA20 + 3H | 3+      | 707.774 | 25.6 ± 0.6          |
| DiazPC + H   | 1+      | 788.592 | 47.7 ± 0.1          |
| DOPC + H     | 1+      | 786.600 | 49.2 ± 0.1          |
| PSPC + H     | 1+      | 762.599 | 50.1 ± 0.1          |
| DiazPC – N₂ + H₂O + H | 1+ | 778.595 | 44.2 ± 0.2 |
| DiazPC – N₂ + DOPC + 2H | 2+ | 773.592 | 63.9 ± 0.3 (A) |
|              |        |         | 65.1 ± 0.3 (B)     |
| DiazPC – N₂ + LAVA20 + 3H | 3+ | 960.969 | 47.4 ± 0.3 (A) |
|              |        |         | 48.4 ± 0.2 (B)     |
|              |        |         | 49.1 ± 0.2 (C)     |
|              |        |         | 50.1 ± 0.2 (D)     |

**Table S3.** Theoretical MS data of fragment ions (b ions and y ions) of LAVA20.

| b ion | y ion | Acetyl     | Amided   |
|-------|-------|------------|----------|
| 100.0393 | G  | 1         | 2078.3001 |
| 228.1343 | K  | 2         | 2021.2786 |
| 356.2292 | K  | 3         | 1893.1837 |
| 469.3133 | L  | 4         | 1765.0887 |
| 540.3504 | A  | 5         | 1652.0046 |
| 639.4188 | V  | 6         | 1580.9675 |
| 710.4559 | A  | 7         | 1481.8991 |
| 809.5244 | V  | 8         | 1410.8620 |
| 880.5615 | A  | 9         | 1311.7936 |
| 993.6455 | L  | 10        | 1240.7565 |
| 1064.6826 | A | 11        | 1127.6724 |
| 1163.7511 | V | 12        | 1056.6353 |
| 1234.7882 | A | 13        | 957.5669  |
| 1347.8722 | L | 14        | 886.5298  |
| 1418.9093 | A | 15        | 773.4457  |
| 1531.9934 | L | 16        | 702.4086  |
| 1718.0727 | W | 17        | 589.3245  |

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Table S4. Theoretical peak table of LAVA20 fragmentation.

| m/z  | Formula               | Peak of                | Isotopic Cluster | Intensity (ppm) |
|------|-----------------------|------------------------|------------------|-----------------|
| 72.0444 | 426.2711               | AVALA                  | a1 = NH3         | 948.6241        |
| 72.0808 | 426.2711               | AVALA                  | a1 = NH3         | 1311.7936       |
| 84.0808 | 426.3075               | LAVA28                 | b2 = LALWW       | 948.6241        |
| 86.0964 | 426.3075               | LAVA28                 | b2 = LALWW       | 1319.8773       |
| 100.0939 | 426.3075              | AVALA28               | y2 = LALWW       | 954.5560        |
| 101.1073 | 426.2245              | AVALA28               | y2 = LALWW       | 1330.9457       |
| 126.0913 | 440.3231              | VALAL-28              | a2 = LALWW       | 957.5669        |
| 129.1022 | 440.3231              | VALAL-28              | a2 = LALWW       | 1334.8559       |
| 143.1179 | 441.3184              | LAVA28                 | y4 = NH3         | 964.6554        |
| 143.1179 | 441.3184              | LAVA28                 | y4 = NH3         | 1336.7776       |
| 157.1335 | 454.3024              | LAVAY                  | y4 = NH3         | 976.6190        |
| 157.1335 | 454.3024              | LAVAY                  | y4 = NH3         | 1362.8508       |
| 164.1288 | 452.2657              | AVALA28               | y5 = LALWW       | 982.5509        |
| 164.1288 | 452.2657              | AVALA28               | y5 = LALWW       | 1390.9144       |
| 171.1128 | 456.2969              | LALW28                 | x1 = LALWW       | 983.5461        |
| 171.1128 | 456.2969              | LALW28                 | x1 = LALWW       | 1393.8355       |
| 172.1081 | 456.2551              | LAVAY                  | x5 = LALWW       | 990.6346        |
| 172.1081 | 456.2551              | LAVAY                  | x5 = LALWW       | 1404.9665       |
| 183.1128 | 466.3024              | LAVA28                 | y5 = LALWW       | 1007.6612       |
| 183.1128 | 466.3024              | LAVA28                 | y5 = LALWW       | 1415.9348       |
| 185.1285 | 468.3180              | LAVAY                  | y6 = LALWW       | 1024.5978       |
| 185.1285 | 468.3180              | LAVAY                  | y6 = LALWW       | 1433.8460       |
| 185.1285 | 468.3180              | LAVAY                  | y6 = LALWW       | 1434.8460       |
| 192.1399 | 469.3133              | LAVAY                  | x6 = LALWW       | 1035.6925       |
| 192.1399 | 469.3133              | LAVAY                  | x6 = LALWW       | 1446.8413       |
| 214.1442 | 469.3501              | AVALE28               | x5 = LALWW       | 1046.8587       |
| 214.1442 | 469.3501              | AVALE28               | x5 = LALWW       | 1454.9308       |
| 214.1442 | 469.3501              | AVALE28               | x5 = LALWW       | 1455.9308       |
| 220.1455 | 470.3602              | LAVAY                  | y7 = LALWW       | 1064.6826       |
| 220.1455 | 470.3602              | LAVAY                  | y7 = LALWW       | 1507.8784       |
| 228.1707 | 471.4083              | LAVAY                  | y7 = LALWW       | 1514.9669       |
| 228.1707 | 471.4083              | LAVAY                  | y7 = LALWW       | 1515.9669       |
| 229.2023 | 472.4920              | LAVA28                 | y8 = LALWW       | 1067.6400       |
| 229.2023 | 472.4920              | LAVA28                 | y8 = LALWW       | 1520.9352       |
| 230.1288 | 511.3602              | AVALE28               | x6 = LALWW       | 1084.6874       |
| 230.1288 | 511.3602              | AVALE28               | x6 = LALWW       | 1550.8831       |
| 240.1707 | 515.3545              | KKLAVA28              | y7 = x6          | 1097.6612       |
| 240.1707 | 515.3545              | KKLAVA28              | y7 = x6          | 1548.9301       |
| 242.1499 | 516.4192              | AVALE28               | y7 = x6          | 1549.9301       |
| 242.1499 | 516.4192              | AVALE28               | y7 = x6          | 1550.9301       |
| 243.1452 | 523.3299              | AVALE28               | b2 = LALWW       | 1090.7501       |
| 243.1452 | 523.3299              | AVALE28               | b2 = LALWW       | 1580.9675       |
| 256.1656 | 536.3602              | KKLAVA28              | y8 = x6          | 1096.6400       |
| 256.1656 | 536.3602              | KKLAVA28              | y8 = x6          | 1551.9934       |
| 256.2020 | 535.3935              | KKLAVA28              | y8 = x6          | 1552.9934       |
| 256.2020 | 535.3935              | KKLAVA28              | y8 = x6          | 1553.9934       |
| 257.1972 | 535.3935              | KKLAVA28              | y8 = x6          | 1554.9934       |
| 258.2327 | 535.3935              | KKLAVA28              | y8 = x6          | 1555.9934       |
| 270.1812 | 535.3935              | KKLAVA28              | y8 = x6          | 1556.9934       |
| 270.1812 | 535.3935              | KKLAVA28              | y8 = x6          | 1557.9934       |
| 270.2376 | 527.3340              | KKLAVA28              | y8 = x6          | 1558.9934       |
| 270.2376 | 527.3340              | KKLAVA28              | y8 = x6          | 1559.9934       |
| 284.1969 | 529.2922              | KLAVA28               | y8 = x6          | 1107.7612       |
| 284.1969 | 529.2922              | KLAVA28               | y8 = x6          | 1606.9468       |
| 284.1969 | 539.3552              | KKLAVA28              | y8 = x6          | 1619.9672       |
Figure S8. Fragment ion mass spectrum of cross-linked product at \( m/z \) 960.632 at \( t_R = 48.8 - 49.2 \) min (peak C of Figure 4e, main article). DPG, 3-desoxy-1-palmitoyl-glycerol; P, precursor; #, [fragment ion – phosphocholine]; §, [fragment ion – 1-palmitoyl-sn-glycero-3-phosphocholine].

Figure S9. Fragment ion mass spectrum of cross-linked product at \( m/z \) 960.632 at \( t_R = 49.8 - 50.2 \) min (peak D of Figure 4e, main article). DPG, 3-desoxy-1-palmitoyl-glycerol; P, precursor; #, [fragment ion – phosphocholine]; §, [fragment ion – 1-palmitoyl-sn-glycero-3-phosphocholine]. The y-type ions marked in light blue result from overlapping with peak C (compare to Figure S8).
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