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Supplementary Material

Grapevine stilbenoids as natural food preservatives: calorimetric and spectroscopic insights on the interaction with model cell membranes

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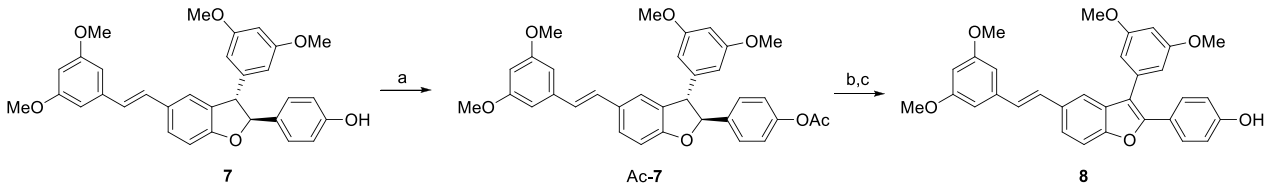
2. Materials and methods

2.1. Materials

*Synthesis of compound **8***



a) Ac_2O, TEA, DCM, rt, overnight, 92%; b) DDQ, DCM, reflux, 48h, 84%; c) KOH, MeOH, 1h, 0°C, 70%;

Scheme S1. Synthesis of compound **8**.

To a solution of **7** (300 mg, 0.587 mmol, 1 eq) in dry DCM (3.5 mL), TEA (245 µL, 1.761 mmol, 3 eq) and Ac_2O (83 µL, 0.881 mmol, 1.5 eq) were added and the resulting solution was stirred overnight (Scheme S1). The reaction mixture was diluted with DCM (5 mL) and washed with water (10 mL). The aqueous layer was extracted with DCM (2 × 15 mL). The combined organic layers were washed with aq 1M HCl (3 × 20 mL), aq 5% $NaHCO_3$ (40 mL), brine (40 mL), dried over anhydrous Na_2SO_4, filtered and evaporated. **Ac-7** was obtained as a white sticky solid in 95% yield and was used as such for the next step. R_f: 0.40 (cyclohexane/EtOAc 7:3). To a suspension of **Ac-7** (280 mg, 0.507 mmol, 1 eq) in toluene (35 mL), DDQ (2.3 g, 10.133 mmol, 20 eq) was added and the mixture was stirred at 40 °C for 48 h. The solvent was evaporated, and the residue was partitioned between DCM and water. The red aqueous phase was extracted with DCM three times. The combined organic phases were washed with aq $NaHCO_3$ 5% three times, then the aqueous layers were extracted with DCM twice. The collected organic phases were dried over anhydrous Na_2SO_4, filtered and evaporated. R_f: 0.51 (cyclohexane/EtOAc 6:4). The obtained crude product (280 mg, 0.507 mmol, 1 eq) was dissolved in THF (4 mL) and MeOH (8mL). A solution of KOH 85% (101 mg, 1.521 mmol, 3 eq) was added at 0 °C and the mixture was stirred at the same temperature for 1h. The reaction mixture was quenched with aq 0.1 N HCl (20 mL). Organic solvents were evaporated and the aqueous phase was extracted with EtOAc three times. The combined organic layers were dried over anhydrous Na_2SO_4, filtered and evaporated. The residue was purified on silica gel by column chromatography, using as eluent Ciclohexane/ EtOAc 7:3 to afford the desired product **8** as a foamy white solid. R_f: 0.34 (cyclohexane/EtOAc 7:3); m.p.: 173-175°C; ^{1}H NMR (600 MHz, C*D*Cl_3) δ (ppm): 7.63 – 7.55 (m, 3H), 7.51 (s, 2H), 7.18 (d, J = 16.2 Hz, 1H), 6.83 – 6.80 (2H, m), 6.68 (d, J = 2.2 Hz, 2H), 6.67

(d, *J* = 2.2 Hz, 2H), 6.55 ((t, *J* = 2.2 Hz, 1H), 6.40 (t, *J* = 2.2 Hz, 1H), 3.84 (s, 6H), 3.81 (s, 6H); ^{13}C NMR (150 MHz, C*D*Cl$_3$) δ (ppm): 161.3 (×2C), 160.9 (×3C), 155.9, 153.6, 151.3, 139.6, 134.8, 132.4, 130.8, 129.5, 128.7 (×2C), 127.5, 123.3, 123.1, 117.9, 116.0, 115.4 (×2C), 111.1, 107.7 (×2C), 104.4 (×2C), 99.9, 55.5 (×2C), 55.4 (×2C).

2.3. Thermal analysis measurements

For the investigation of stilbenoid-containing membranes, replicates for the several SUVs dispersions were prepared and analysed also in order to consider the variability and reproducibility of the liposome self-assembly process carried out by means of the preparation protocol reported in the Materials and Methods section of the main text. An example showing the micro-DSC scans performed on two replicates of the 2:3 DPPC:DSPC SUVs dispersions is reported in Figure S1.

We observe that the replicates' profiles are almost superimposable and only low levels of variability in the liposomes self-assembly process occur. Moreover, the differences are not significant, and the replicates involve the same enthalpy variation upon the gel-to-liquid crystalline phase transition.

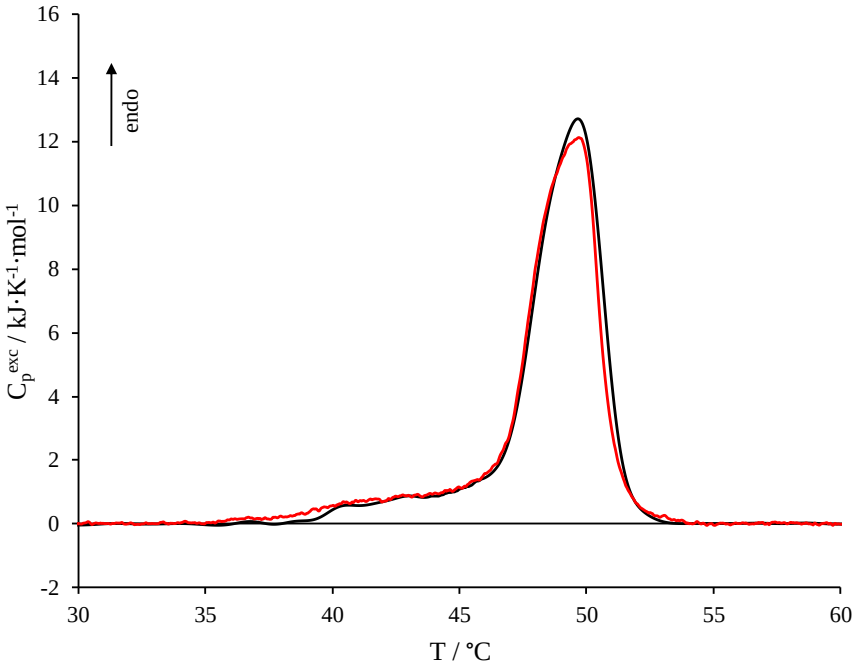


Figure S1. Example reporting the micro-DSC thermograms for two replicates of DPPC:DSPC 2:3 SUVs dispersions.

As far as the influence of the lipid:resveratrol molar ratio is concerned, we verified whether the difference produced on the micro-DSC thermograms (and hence antimicrobial activity) by monomers and dimers might have been merely ascribable to a "concentration" issue, or in other words if the effects generated by a certain dimer concentration might be comparable to double concentration of the respective monomer. For this purpose, we chose to compare resveratrol **1** (monomer) and (±)-*trans*-δ-viniferin **3** (δ-dimer) as an example, and specifically to investigate a 10:2 versus 10:1 lipid:resveratrol molar ratio. The micro-DSC output obtained is reported in Figure S2.

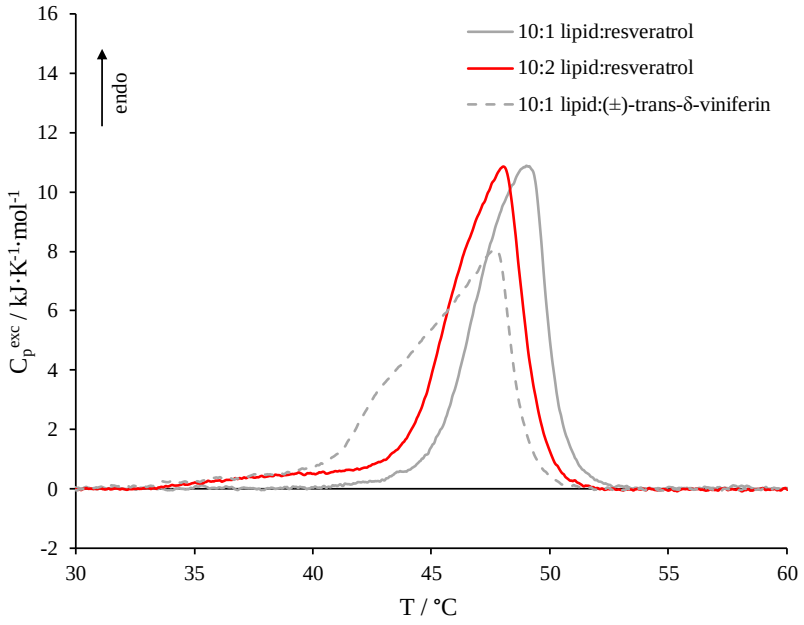


Figure S2. Micro-DSC thermogram for DPPC:DSPC 2:3 vesicles incorporating resveratrol **1** as 10:2 lipid:resveratrol molar ratio (red trace). The already shown profiles of 10:1 lipid:resveratrol and 10:1 lipid:(±)-*trans*-δ-viniferin(**3**) are reported for a better comparison as solid grey trace and dashed grey trace, respectively.

We observe that a double concentration of resveratrol **1** (red curve) does not produce the same effects of resveratrol dimer **3**. Indeed, the membrane is entropically destabilized and the calorimetric profile results to be only shifted towards lower temperatures without the formation of the severe phase separation caused by the dimer. Therefore, we may assess that the modification in membrane thermodynamic stability provided by the dimers **3** is mainly due to the lower hydrophilic/lipophilic balance and only partially to the different size.

A further information that arises from these measurements is about the connection between the lipid:stilbenoid molar ratio and the magnitude of the effects given by the compounds. In particular, we observe that the influence of resveratrol is directly proportional to its concentration, revealing

that, at 10:1 lipid:stilbenoid molar ratio, the effects of the compounds have not reached a saturation level yet.

As concerns the stilbenoid-membrane interaction, in order to evaluate the insertion process of three specific stilbenoids into the vesicle phospholipid bilayer (dehydro-δ-viniferin **6**, (±)-pterostilbene *trans*-dihydrodimer **7** and dehydro-pterostilbene dimer **8**, shown in the main text), additional micro-DSC measurements were performed on samples prepared by adding and well mixing a volume of 1 μL stilbenoids as highly concentrated DMSO solutions with 1 mL of dispersion containing the preformed reference liposomes (2:3 DPPC:DSPC SUVs at 2.8 mM phospholipid concentration) just before launching the measurement.

Although many works in the literature already report that no significant effects are produced by very small DMSO molar fractions ($\chi_{DMSO} < 0.005$) on the thermotropic behaviour of phospholipid liposomes (Bonora, S., *et al. Thermochimica Acta* 433.1-2 (2005): 19-26; Ricci, M., *et al. Biochimica et Biophysica Acta (BBA)-Biomembranes* 1858.12 (2016): 3024-3031), we tested the influence of the addition 1 μL DMSO to 1 mL of 2:3 DPPC:DSPC SUVs dispersion on the resulting calorimetric profile, and the outputs are shown in Figure S3 (in our case, the final buffer/DMSO mixture has an average $\chi_{DMSO} = 0.0003$).

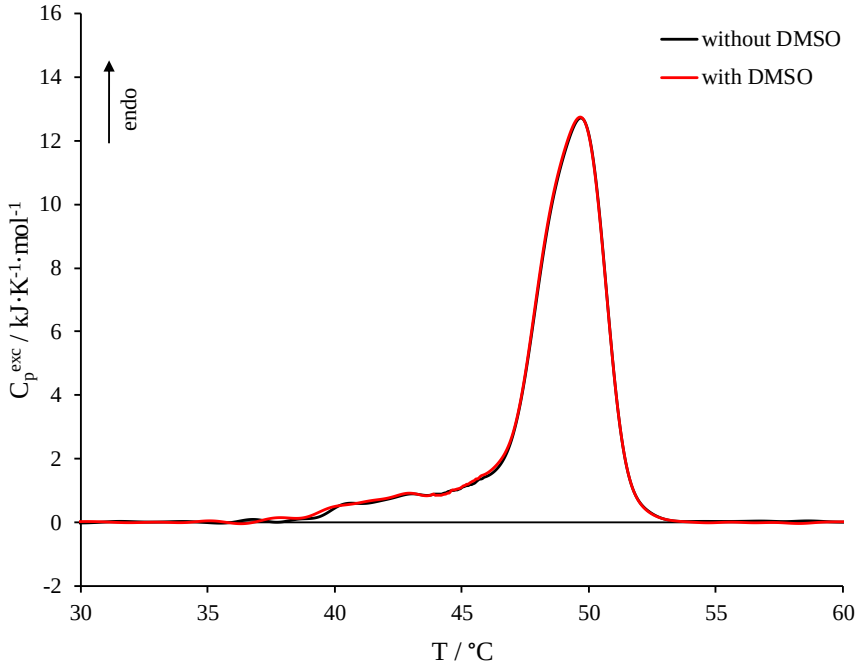


Figure S3. Micro-DSC thermograms for DPPC:DSPC 2:3 vesicles without (black trace) and with (red trace) the external addition of DMSO (final buffer/DMSO mixture with average $\chi_{DMSO} = 0.0003$) to SUVs dispersion.

We observe that the gel-to-liquid crystalline phase transition represented by the calorimetric profile remains unaffected upon the presence of such a DMSO % v/v concentration in buffer, hence allowing a correct calorimetric investigation of the stilbenoid-containing samples specified above (all the DMSO-containing samples were prepared starting from the same batch of DPPC:DSPC 2:3 SUVs preparation).

3. Results and discussion

3.1. Differential scanning calorimetry

As far as the several stilbenoid-membrane interactions are concerned, in the following Figure S4 we report the micro-DSC thermograms for membrane containing the considered stilbenoid monomers, dimers and dehydro-dimers for a better direct comparison.

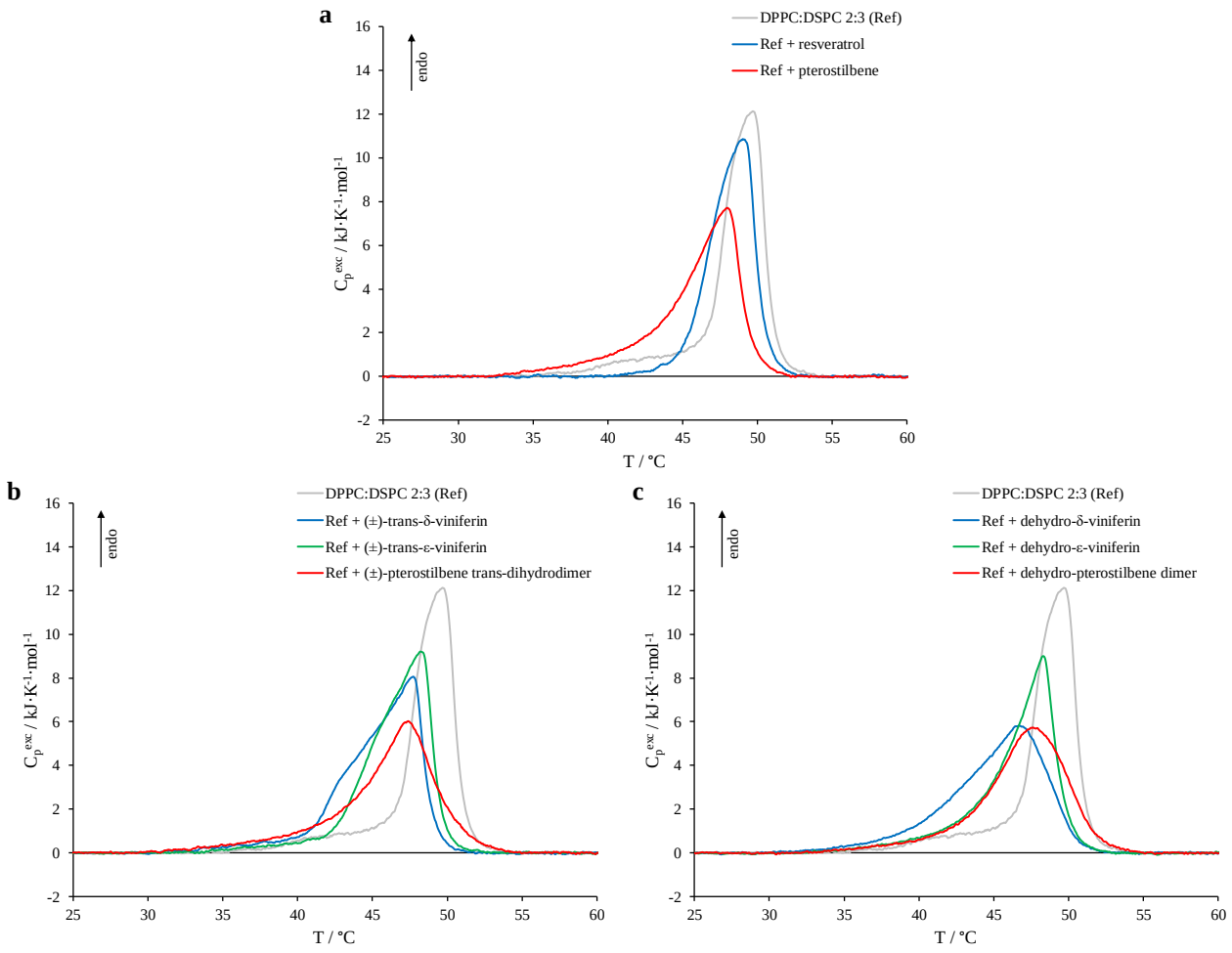


Figure S4. Micro-DSC thermograms for DPPC:DSPC 2:3 vesicles alone (reference grey traces) and incorporating stilbenoid a) monomers (resveratrol **1** in blue and pterostilbene **2** in red), b) dimers ((±)-*trans*-δ-viniferin **3** in blue, (±)-*trans*-ε-viniferin **4** in green and pterostilbene *trans*-dihydrodimer **7** in red), and c) dehydro-dimers (dehydro-δ-viniferin **6** in blue, dehydro-ε-viniferin **5** in green and dehydro-pterostilbene dimer **8** in red).

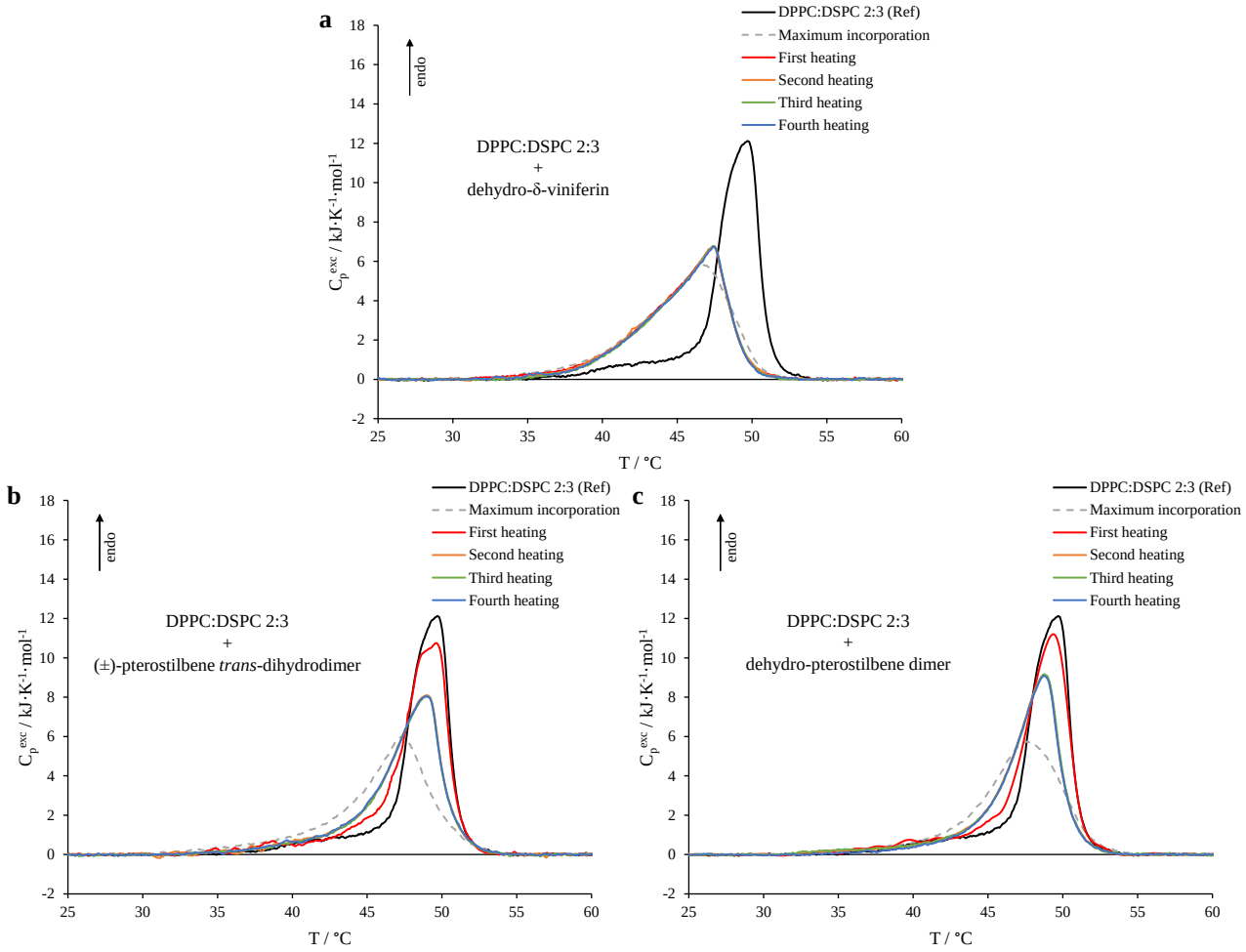


Figure S5. Micro-DSC thermograms obtained for DPPC:DSPC 2:3 vesicles with the external addition of a) dehydro-δ-viniferin **6**, b) (±)-pterostilbene *trans*-dihydrodimer **7** and c) dehydro-pterostilbene dimer **8** from DMSO solutions. All four heating scans applied are consecutively reported as red, orange, green and blue traces. The profiles obtained for DPPC:DSPC 2:3 vesicles and for those with the maximum incorporation of the respective stilbenoids are also reported from Figure 2 of the main text for the sake of comparison (black solid curve and grey dashed one, respectively).

Figure S5 reports the micro-DSC thermograms obtained for DPPC:DSPC 2:3 vesicles with the external addition of dehydro-δ-viniferin **6** (panel *a*), (±)-pterostilbene *trans*-dihydrodimer **7** (panel *b*) and dehydro-pterostilbene dimer **8** (panel *c*) from DMSO solutions. All four heating scans applied are reported in order to better describe the insertion process of the three compounds into the bilayer.

As reported in the main text, the addition of the compounds was performed at room temperature, *i.e.* at a temperature in which the model membrane is in the "gel" phase. Instead, real cell membranes at physiological conditions may present regions that are already in the "liquid crystalline" phase, whose properties might allow an easier insertion of the compounds. For this reason, in order to

provide the compounds also with a membrane in the liquid crystalline phase, as well as to ensure the absence of metastable lipid phases, several heating/cooling cycles were performed.

We observe that the external addition of **6** to the model membrane immediately results in similar effects to those achieved by the direct mixing of the compound with phospholipids (Figure 3a). Indeed, already the first heating scan (red trace) is superimposable to the one obtained with the maximum incorporation (discussed in the main text and reported here as a grey dashed trace for the sake of comparison), as are the following three scans, confirming the absence of metastable lipid phases. On the other hand, the external addition of both the (±)-pterostilbene *trans*-dihydrodimer **7** and the dehydro-pterostilbene dimer **8** lead to no significant effects on the thermotropic behaviour of the model membrane at the first scan (red traces in Figure S5b-c), revealing the missed insertion of the compounds into the bilayer. Only at the second heating (at which stable lipid phases were achieved, as revealed by the overlap of the third and fourth scans to the second ones) we observe a slight entropic destabilization of the membrane thermodynamics as well as an overall enthalpic contribution of (40 ± 2) $kJ \cdot mol^{-1}$ for both systems, but the calorimetric profiles achieved are completely different to the ones obtained with the maximum incorporation (grey dashed traces in Figure S5b-c). Such a result reflects that both **7** and **8** provide the membrane with different and weaker destabilizing effects, likely linked to only a partial insertion of the compounds into the bilayer or, in any case, to a more superficial interaction. However, we would like to underline here that this slight destabilizations gained at the second heating scans could have also been simultaneously helped by the high mobility of the phospholipid molecules because of thermal excitation since a complete heating up 80 °C was carried out, a condition that does not happen physiologically.

Therefore, such considerations do not modify the overall picture emerging from the first heating scans (which are considerable as a first shot of the stilbenoid-membrane interaction), explaining the reason for which a potentially good antimicrobial agent as **7** is characterized by so high MIC and MBC values (Mattio, L. M., *et al. Scientific Reports*, 9.1 (2019): 1-13).

3.2. NMR spectroscopy

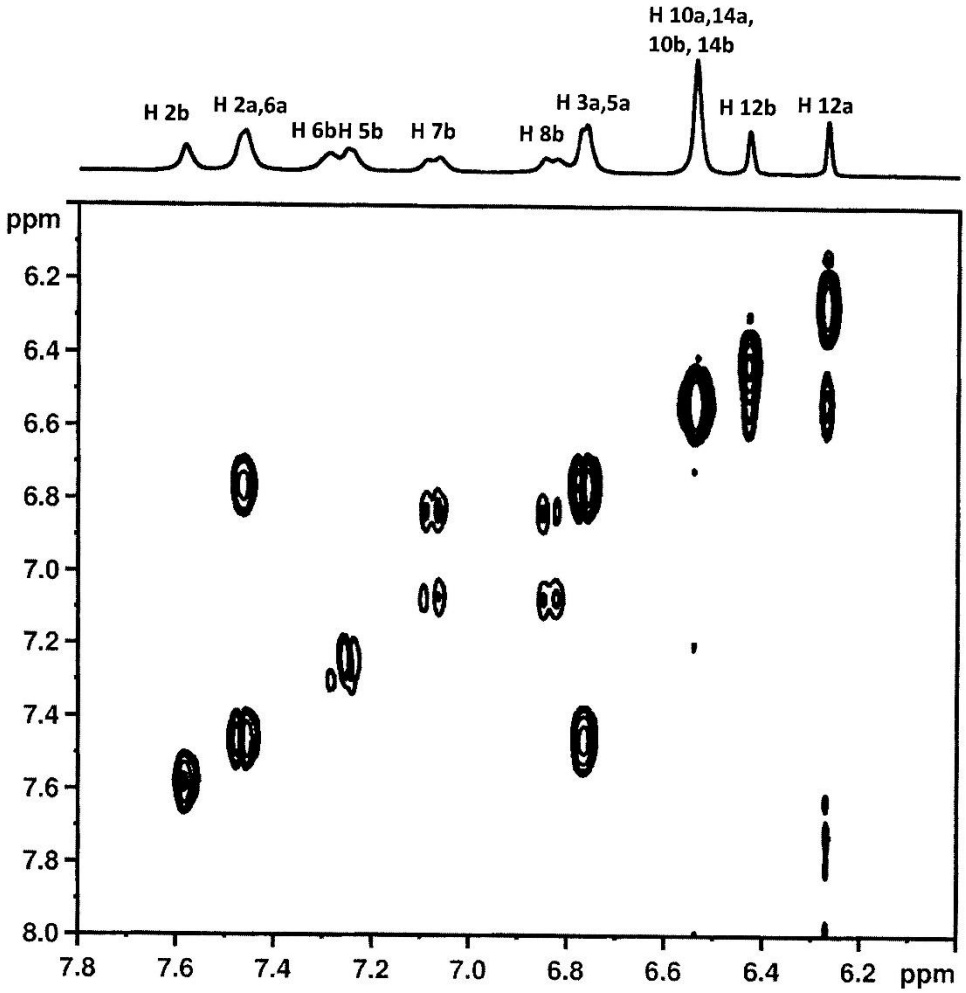


Figure S6. 2D COSY spectrum of dehydro-δ-viniferin **6** trapped within SDS micelles.

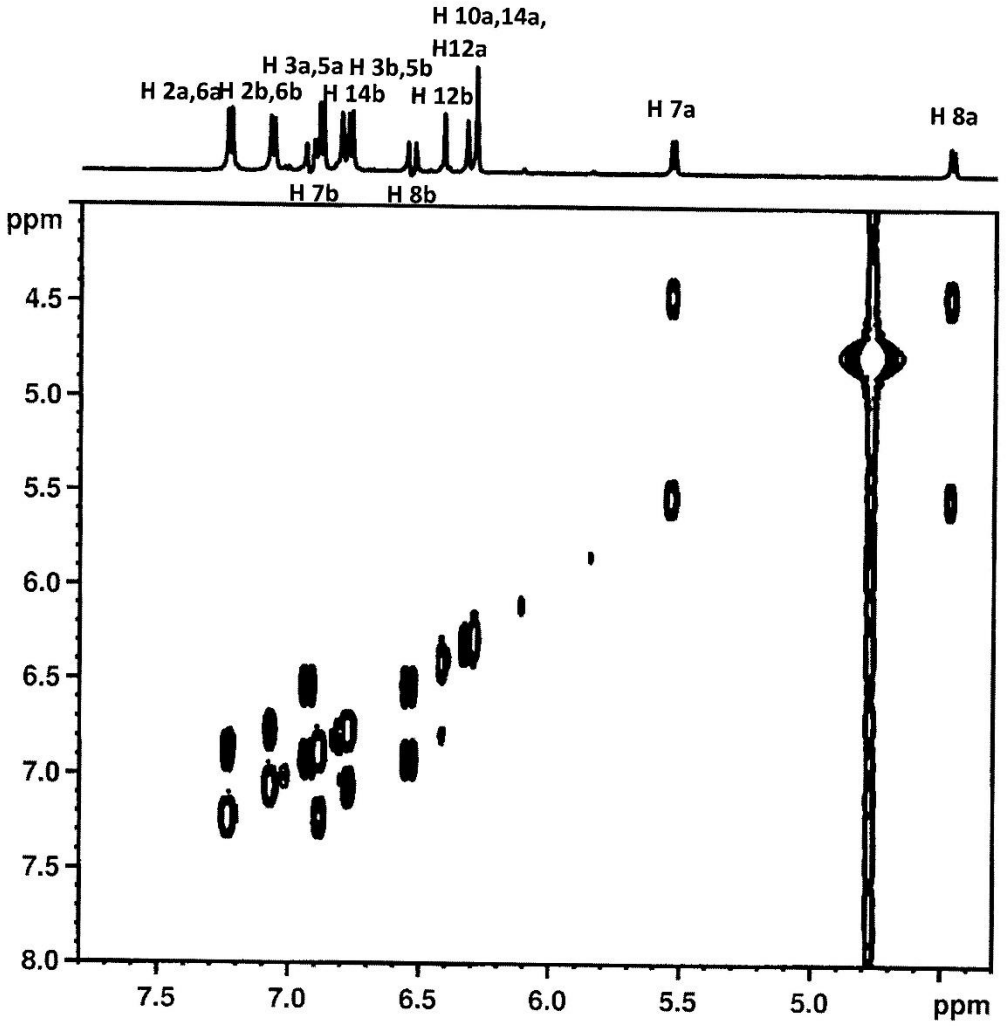


Figure S7. 2D COSY spectrum of (±)-*trans*-ε-viniferin **4** trapped within SDS micelles.

Table S1. ^{1}H chemical shift values (ppm) for resveratrol **1**, dehydro-δ-viniferin **6** and (±)-*trans*-ε-viniferin **4.**

position	**1**	**position**	**6**	**position**	**4**
2	6.69	2a	7.46	2a	7.23
2’	7.55	3a	6.76	3a	6.89
3’	6.95	5a	6.76	5a	6.89
4	6.36	6a	7.46	6a	7.23
5’	6.95	10a	6.53	7a	5.55
6’	7.55	12a	6.27	8a	4.47
6	6.69	14a	6.53	10a	6.30-6.33
α	7.15	2b	7.59	12a	6.30-6.33
α’	7.00	5b	7.22	14a	6.30-6.33
		6b	7.28	2b	7.07
		7b	7.07	3b	6.77
		8b	6.84	5b	6.77
		10b	6.53	6b	7.07
		12b	6.43	7b	6.94
		14b	6.53	8b	6.53
				12b	6.41
				14b	6.80