One-step iodination of the diazocyclopentadien-2-ylcarbonyl group—a new and convenient preparation of effective radiolabelled photoaffinity probes

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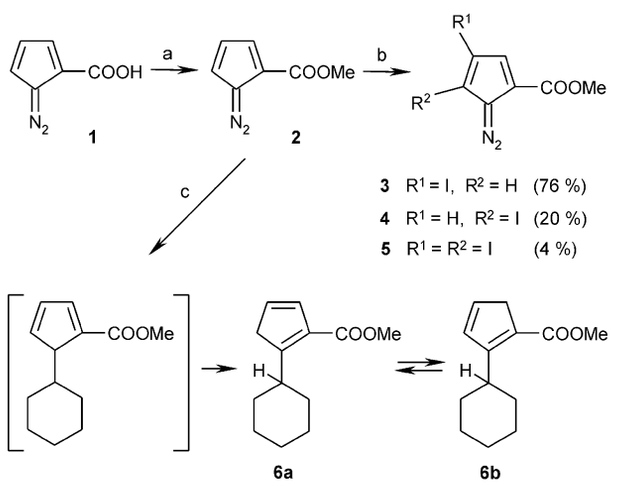
A detailed study devoted to direct iodination of the photoactivatable diazocyclopentadien-2-ylcarbonyl (Dcp) group is presented. The iodination does not influence the high carbene reactivity of the Dcp-generated carbene. It was shown that the Dcp substituent forms 4-mono-, 5-mono- and 4,5-diiododerivatives upon iodination under oxidative conditions (76, 20 and 4%, respectively, when DcpOMe **2** is iodinated). Photolysis of the individual products of iodination in cyclohexane resulted in rather high insertion into non-activated CH bonds, without noticeable loss of iodine. Syntheses of new phospholipid and ganglioside membrane probes are also described which incorporate the Dcp function *via* a labile ester bond. A [^{125}I]-Dcp-phosphatidylcholine probe exhibiting high specific radioactivity (~500 Ci mmol^{-1}) was easily prepared at yields of 90% (on the starting Na^{125}I), by using peracetic acid as an oxidant. Furthermore, it was successfully used for photolabelling of the integral protein hemagglutinin in a well-characterised influenza virus model. In summary, the Dcp group is efficient for labelling a wide variety of molecules, and as such, it provides a new tool for exploring a diverse range of biological systems.

Introduction

Because photoaffinity labelling (PAL) provides direct evidence of spatial proximity of molecules, it is a widely used method in studies of structure and function of biological systems.[1] The photochemically labile groups incorporated in a natural ligand are converted by irradiation into highly reactive intermediates (carbene, nitrene or biradical), which are capable of forming a covalent bond with the nearest fragment of a biomolecule. However, as with any probing technique, PAL is not deprived of shortcomings, and improvements are needed. Therefore, the search for new photoaffinity groups with a wide range of criteria is required, these criteria include high reactivity, a short lifetime of the excited species, biocompatible irradiation conditions, avoiding intramolecular rearrangement into less reactive intermediates after photolysis, minimal size, stability of covalent bonds with target molecule, possibility of the reliable detection of cross-linking products, dark stability, and last but not least availability.[1] None of the hitherto known labels fulfil all requirements. Recently, experiments performed to test the efficiency of PAL for a number of labels for peptides[2] and DNA[3] demonstrated the superiority of the carbene-generating 3-(trifluoromethyl)-3-phenyldiazirine (TFD) group, that was originally developed by Brunner and co-workers.[1c]

To enhance the sensitivity of PAL, γ- and strong β-emitters (^{125}I, ^{131}I, ^{32}P *etc.*) are usually used. However, probes bearing such isotopes exhibit a short shelf life. A solution to this problem would be to synthesise their precursors that can be stored for a long time, and then perform radiolabelling immediately before use. In order to facilitate analysis of the PAL products, it is also desirable to introduce a radioisotope into the photoaffinity group itself. The electrophilic substitution with ^{125}I under oxidative conditions for aromatic residues activated with any electron-donor substituent is well-known.[4] For a number of photoaffinity groups, *e.g.*, fluorophenyl azide,[5] TFD,[6] and benzophenone,[7] functionalisation methods were developed that allow the introduction of ^{125}I, although the syntheses of the probes then becomes significantly more complex. Obviously, this is the reason why to this day, the most commonly used probes for PAL are derivatives of commercially available aryl azides that can be easily iodinated under oxidative conditions. However, it is well documented that nitrenes generated by aryl azides upon photolysis, undergo rapid ring expansion to long-lived dehydroazepines which are most reactive with nucleophiles, such as amine and thiol residues.[1]

The carbene-generating diazocyclopentadien-2-ylcarbonyl (Dcp) group was suggested for PAL by Nielsen *et al.*,[8a] with especial application to studies of biological membranes rich in non-activated hydrocarbon residues. This small-sized and available labelling group appears attractive to us: DcpOH **1** (Scheme 1) is prepared by a simple 3-step synthesis and, in the absence of UV light, is rather stable under a range of physicochemical



Scheme 1 a) CH_2N_2; b) NaI (10 equiv.), chloroamine T (12 equiv.), MeOH; c) *hν*, $\lambda > 300$ nm, 3 min, cyclohexane.

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Table 1 ^{1}H NMR (500 MHz, C_6D_{12}, 25 °C, TMS; δ/ppm, J/Hz) and MS (EI, 70 eV) data for the compounds **2**–**5**

Compound	H-3	H-4	H-5	CH_3	*m/z* (%)
2	6.62 dd (1H) $J_{3,4}$ = 3.15 $J_{3,5}$ = 1.95	5.91 dd (1H) $J_{4,5}$ = 4.64 $J_{4,3}$ = 3.15	6.79 dd (1H) $J_{5,3}$ = 1.95 $J_{5,4}$ = 4.64	3.69 s (3H)	149 (100, $M^+ - H$), 121 (40, $M^+ - H - N_2$); $C_7H_6N_2O_2$ (M^+) requires 150.0469
3	6.66 d (1H) $J_{3,5}$ = 2.20	—	6.91 d (1H) $J_{5,3}$ = 2.20	3.70 s (3H)	276 (90, M^+), 233 (100, $M^+ - N_2 - Me$); $C_7H_5N_2IO_2$ (M^+) requires 275.9367
4	6.51 d (1H) $J_{3,4}$ = 3.42	6.17 d (1H) $J_{4,3}$ = 3.42	—	3.70 s (3H)	276 (85, M^+), 233 (100, $M^+ - N_2 - Me$); $C_7H_5N_2IO_2$ (M^+) see above
5	6.70 s (1 H)	—	—	3.70 s (3 H)	402 (62, M^+), 359 (90, $M^+ - N_2 - Me$); $C_7H_4N_2I_2O_2$ (M^+) requires 401.8333

conditions, such as heat (>150 °C), mild bases and acids (including peracetic acid),[8b] and oxidising conditions. We synthesised $^{14}C/^3H$-labelled phospholipid probes with the Dcp function localised at the ω-position of a fatty acyl chain. Photolabelling yielded carbene insertion into CH bonds of cyclohexane or constituents of model membranes upon photolysis at $\lambda > 300$ nm.[9] The probes were applied in studies of the membrane topography of cytochrome P-450.[10] In further studies of the Dcp group properties, we have found that it can be iodinated easily under oxidative conditions, the ability to generate carbene remaining high.[11] The present work is a detailed study on the iodination of the Dcp function including aspects on its photolysis. We also report on the syntheses of new lipid Dcp-probes, preparation of [^{125}I]-Dcp-phosphatidylcholine of high specific radioactivity, and its application for PAL of the membrane-embedded proteins in the well-characterised influenza virus model.

Results and discussion

Oxidative iodination of the Dcp group, and its photochemical properties

To facilitate the resolution of individual compounds by HPLC, methyl ester **2**, which is the smallest Dcp probe was used in a study of the iodination products (Scheme 1). Previously, we used chloroamine T (CAT) fixed on the polystyrene surface (Iodo-Beads®, Pierce, U.S.A.)[4b] for iodination of the Dcp ester **11** (Scheme 3).[11] But in the case of hydrophobic substances, most of the product remained adsorbed on the polymeric matrix, even in methanol. We have elevated the yield of target product treating ester **2** with excess quantities of NaI and CAT. Subsequent HPLC gave three individual products, 4-mono- (**3**), 5-mono- (**4**) and 4,5-diiododerivatives (**5**) (Scheme 1), characterised by ^{1}H NMR and EI MS (Table 1).

Iodination was accompanied by significant changes of UV spectra: initial ester **2** exhibited λ_{max} at 314 nm (EtOH), whereas iodinated products **3**, **4**, and **5** showed the shifted λ_{max} values of 322, 316, and 328 nm, respectively. Measurements of ε values for **2**–**5** were complicated by their high volatility, but they cannot differ significantly from the corresponding values found for methyl 11-(Dcp-oxy)undecanoate **11** (λ_{max} at 314 nm, ε 15000 in EtOH) and for the mixture of its iodination products (λ_{max} at 326 nm, ε 16700, EtOH).[11] Both acid **1** and Dcp-amino derivatives are iodinated easily giving similar mixtures of mono- and diiododerivatives, irrespective of the molar ratio NaI–Dcp substrate within the 0.5–10 range (data not shown). The capability of diazocyclopentadiene to undergo electrophilic substitution reactions is known,[12a] but, to our knowledge, the direct halogenation of Dcp has not been reported.

The photochemical reactivity of iodinated Dcp is interesting, because heavy atoms are known to promote intersystem crossing from excited singlet states to undesirable triplet states. The subsequent reactions may be restricted to hydrogen abstraction

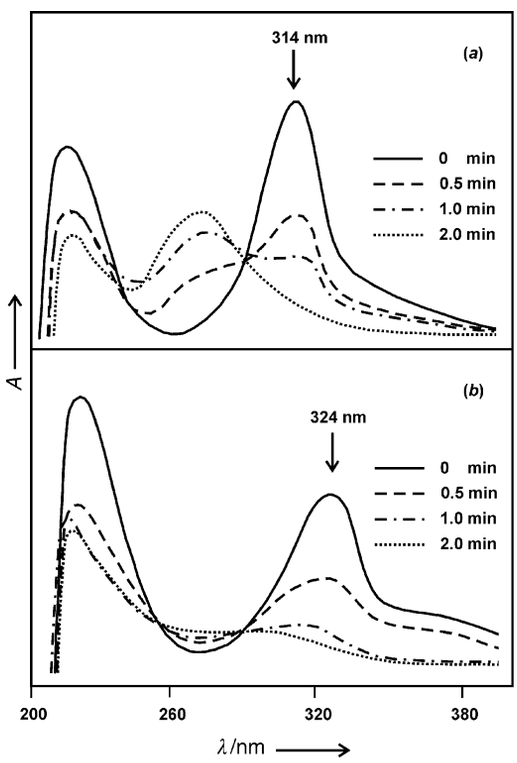


Fig. 1 Photolysis of Dcp-substrates (6 mM) in cyclohexane: (*a*) DcpOMe **2**, $\lambda > 300$ nm; (*b*) iodinated ester **2** (**3** + **4** + **5**), $\lambda > 320$ nm.

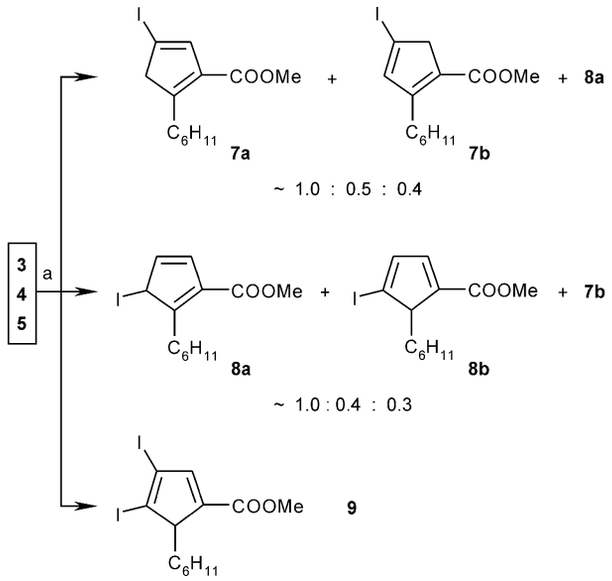
only.[13] Also, of particular importance is the stability of the CI bond. Previously, photodeiodination which reduces the sensitivity of PAL and leads to artefacts connected with unspecific trapping of iodine radicals by biomolecules was observed for some iodoaryl azides.[5,14] In this connection, we had to examine the photochemical properties of the Dcp group itself.

The chemistry of cyclopentadienylidene, carbene generated upon photoactivation of diazocyclopentadiene, appears to be well established. Its ground state is a triplet state, but the chemistry in solution results from the singlet state, and CH insertion proceeds with high efficiency.[12b,c] The course of Dcp photolysis is shown in Fig. 1a. A solution of ester **2** in cyclohexane was irradiated for 3 min. The solution was not concentrated, because the volatility of products might lead to disproportionation losses, instead C_6D_{12} (10% v/v) was added, and ^{1}H NMR with double resonance analysis was performed. TLC showed the most abundant compound was rather non-polar, and also obtained were less polar minor components (their ratio varied depending on the procedure of evaporation and resolution), with a little tar at the start. The ^{1}H NMR spectrum of the mixture remained the same upon storage for several days at 4 °C, thus allowing us to attribute the structures **6a** or **6b** to the only abundant product of photolysis, besides tar (Scheme 1,

Fig. 2). The cyclohexyl proton H1 resonance at a rather low field (δ 3.55 ppm) was completely confirmed by double resonance at the adjacent cyclohexyl protons in high field (δ 1.53 ppm); these spectra were taken in CD_3OD. ESI MS also evidenced a cyclohexane insertion product.

Such endocyclic double bond isomers of substituted cyclopentadienes were reported to be in thermodynamic equilibrium, with transformations proceeding *via* a proton transfer from C-5 to the adjacent C-atom, and proton-cyclopentadienyl anion complexes being probable transitional states.[15b] The structures and ratio of isomers depend mainly on the character of substituent(s),[15b] for methyl cyclopentadienecarboxylate the product is exclusively or very largely the 1,3-diene (in our case structure **6b**).[15a] Only minute tar quantities were seen on TLC, but a comparison of integral resonance intensities in ^{1}H NMR spectra of compound **2** taken prior and after photolysis in cyclohexane (6 mM) showed *ca.* 42% yield of CH insertion product. For a comparison, photolysis of the 3-(trifluoromethyl)-3-phenyldiazirine derivative in cyclohexane gave rise to several compounds, among which the CH insertion product accounted for 58% at an initial concentration of 2 mM, and 28% at 30 mM, as determined by GC analysis.[6] So, we can note that the ability of the Dcp-generated carbene to insert into ordinary CH bond is close to that of the most efficient TFD label.

The photolysis course of iodinated Dcp ester **2** (a mixture of compounds **3**, **4**, and **5**) in cyclohexane was followed with UV spectra (Fig. 1b). To study the products of the photoreaction, individual iodinated esters **3**, **4**, and **5** were photolysed in cyclohexane for 3 min, and then reaction mixtures were analysed as described above for the ester **2**. TLC showed, apart from some tar as polar tails, highly nonpolar products of the carbene cross-linking to cyclohexane (ESI MS data). ^{1}H NMR spectra of reaction products (see Experimental, and Fig. 3 for representative spectra) show well-resolved peaks. The double resonance technique allowed us to propose the following pattern of photoreaction products (Scheme 2). No evidence of isomers of diiodide **9** was found in the spectrum of the photolysis products of ester **5**. Such compounds, having up-field resonances of the ring (substituted) methylene, would be clearly observed. We do not discuss here possible mechanisms of isomeric transformations that lead to formation of compounds **7**–**9**. This will be the issue of further studies. Noteworthy is the absence of signals corresponding to non-iodinated esters **6a** (**6b**), in contrary to iodinated fluorophenyl azides that yield up to 8% (preparative TLC) deiodinated product of CH insertion.[5] A rather high stability of the CI bond in the esters **3**–**5** was confirmed by 20 min photolysis of ^{125}I-labelled 11-(Dcp-oxy)undecanoate **11** in cyclohexane: subsequent careful washing of the reaction mixture with aqueous $NaHSO_3$ extracted less than 3% of the starting radioactivity.[11] These results show that the iodinated Dcp group can be photolysed without considerable loss of iodine. This could be owing to the longer absorption wavelengths of Dcp chromophore, leading to a more efficient photoactivation as compared to that of phenyl azides. The same was suggested by Weber and Brunner regarding the significant photostability of the CI bond in iodinated 3-phenyldiazirines upon photolysis.[6]



Scheme 2 a) *hν*, λ > 320 nm, 3 min, cyclohexane.

A comparison of integral resonance intensities in the ^{1}H NMR spectra of the esters **3**–**5** taken prior and after photolysis in cyclohexane (6 mM) showed that cross-linking products (**7ab**, **8a**), (**8ab**, **7b**) and **9** were formed with the yields of 30, 30, and 26%, respectively. The CH insertion efficiency for the iodinated Dcp function appears to be somewhat lower than that for the non-iodinated one. For a comparison see the data obtained for methyl 4-azido-2-iodo-3,5,6-trifluorobenzoate (12%, by preparative TLC)[5] and for 2-iodo-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyl acetate (48%, by GC/MS)[6] at initial concentrations of 2 mM in cyclohexane. Although further studies are needed to elucidate the details of the photochemistry of the iodinated Dcp group, it may be supposed because of the sufficiently high yields of CH insertion products that, upon photoactivation, this function reacts mostly *via* a singlet carbene.

The existence of the iodinated Dcp label as a mixture of mono- and diiododerivatives is obviously not of importance when PAL products should be analysed at the level of large

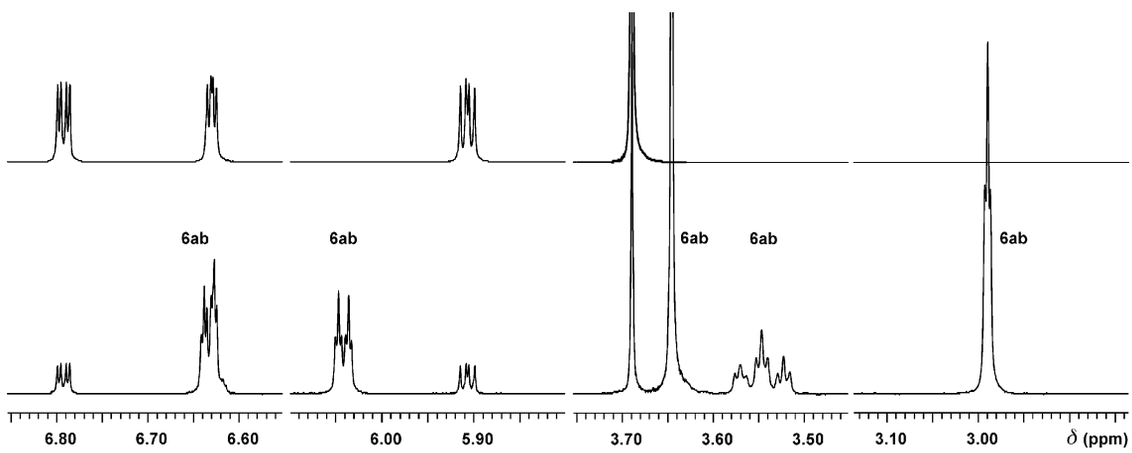


Fig. 2 ^{1}H NMR spectra (C_6D_{12}) of DcpOMe **2** prior (upper row) and after (lower row) photolysis in cyclohexane; photolysis was performed till 80% conversion.

biomolecules, and could be taken into account only if smaller molecules (*e.g.* short peptides, or amino acids) are under study.

Syntheses of phospholipid and ganglioside photoprobes

As already noted, a highly photoactive Dcp group is of particular interest for PAL within the hydrophobic region of biological membranes. Over the last 25 years, numerous photoactivatable lipids have been synthesised and applied in structural and functional studies.[6,9,16] Most of these lipids are phosphatidylcholines and sphingolipids (particularly, gangliosides) that carry a photoaffinity group linked through amide, ester, or ether function to the ω-position of the fatty acyl chain. A high specific radioactivity of the probes is important in PAL studies of membrane embedded proteins, because a great share of the photoaffinity label immersed into the lipid bilayer is wasted as a result of cross-linking with surrounding lipids.

We have prepared a set of Dcp-labelled photoaffinity lipids for subsequent radioiodination, particularly phosphatidylcholine **12** (PC) and gangliosides **16** and **17** designed for the depth-dependent membrane probing (Scheme 3). To make the analysis of PAL products easier, it is desirable to have a labile bond between photoaffinity group and the rest of the probe molecule. Since PAL deals most often with proteins as target biomolecules, the ester bond, being much more susceptible to alkaline hydrolysis than the peptide, is suitable here. First, the ω-substituted fatty acyl methyl ester **11** was synthesised. 11-Aminoundecanoic acid was deaminated with HNO_2 in water to yield 11-hydroxyundecanoic acid then esterified by methanol, and treated with Tf_2O to give triflate† **10**, which was further reacted with DcpOH **1** to form the ester **11**. By its partial saponification, 11-(Dcp-oxy)undecanoic acid was obtained, and then subjected to esterification of lysophosphatidylcholine following the established procedure,[17] to give Dcp-PC probe **12**.

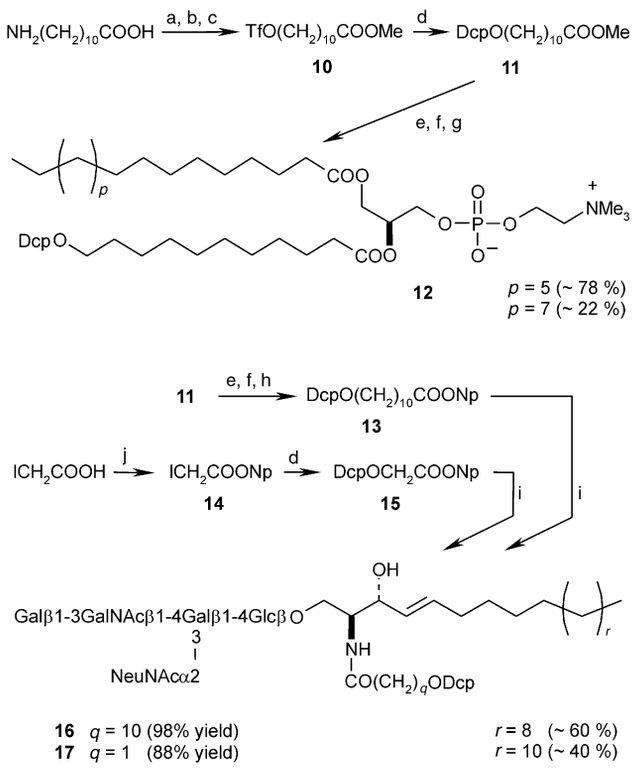
The long-chain ganglioside **16** was synthesised by acylation of lysoganglioside GM1 with *p*-nitrophenyl ester **13** in DMSO. To obtain the short-chain probe **17**, *p*-nitrophenyl ester **15** was prepared, starting from the iodoacetic acid through its condensation with *p*-nitrophenol and subsequent treatment by DcpOH **1**. Notice the high yields of gangliosides **16** and **17**. This might be due to using DMSO as a solvent for the total conversion of lysoganglioside *via* reaction with activated esters **13** and **15**. This is followed by a gel filtration (instead of adsorption or reversed phase chromatography) to purify target products present at micromole quantities.

† The IUPAC name for triflate is trifluoromethanesulfonate.

PAL of the membrane proteins of the influenza virus

We tested the ability of radioiodinated Dcp-labelled phosphatidylcholine [^{125}I]-**12** to probe integral proteins on the well-studied model of influenza virus.[18] The virus envelope is formed by the lipid bilayer and two glycoproteins, hemagglutinin (HA) and neuraminidase (NA), packed in spikes protruding into the outer medium. The HA monomer consists of two SS-bonded subunits: a small one (HA_2) that penetrates deep into the apolar region of the lipid bilayer, and a large polar HA_1. The inner surface of the viral membrane is lined with matrix protein (M_1), which in turn is in contact with the nucleocapside protein (NP). In this study, we used a classical strain of the influenza virus, fowl plaque virus (FPV).[18b,c]

We obtained the probe [^{125}I]-**12** with high specific radioactivity (~500 Ci mmol^{-1}; 90% yield from the starting Na^{125}I) applying peracetic acid as an oxidant.[4c] In contrast, when labelling Dcp-bearing substances with even tracer amounts of iodine



Scheme 3 a) HNO_2; b) MeOH–HCl; c) Tf_2O–Py; d) **1**–Et_3N; e) KOH, *i*PrOH–H_2O; f) HCl; g) lysophosphatidylcholine, DCC, 4-aminopyridine; h) CF_3COONp–Py; i) lysoGM1–Et_3N; j) HONp, DCC.

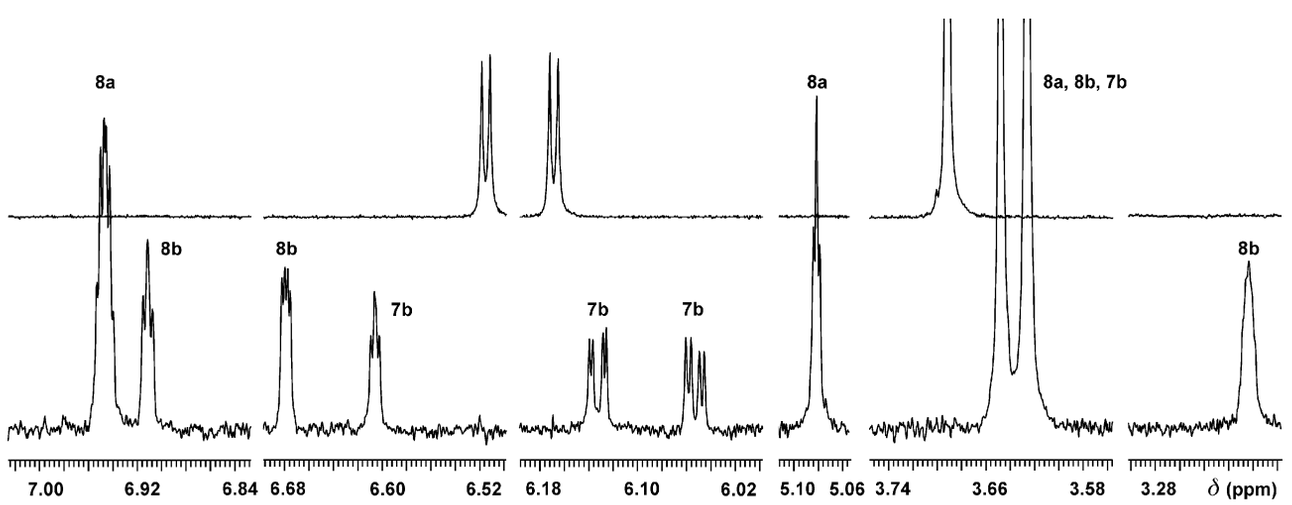


Fig. 3 ^{1}H NMR spectra (C_6D_{12}) of 5-iodo-DcpOMe **4** prior (upper row) and after (lower row) complete photolysis in cyclohexane.

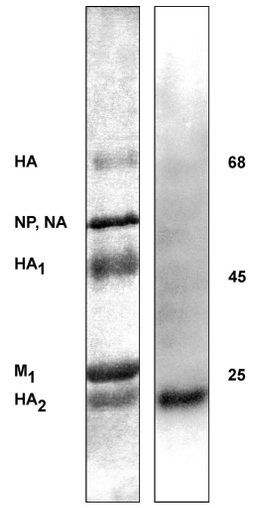


Fig. 4 Photoaffinity labelling of the virus proteins with a Dcp phospholipid probe. Fowl plague virus suspension (0.1 mg protein) was incubated with the probe [^{125}I]-**12** (0.16 nmol, 80 μCi) for 3 h at 37 °C, then irradiated for 5 min at $\lambda > 320$ nm. After delipidation with organic solvents, the material was resolved by 12.5% SDS-PAGE under reducing conditions.[19] Gel was stained with 0.05% Coomassie brilliant blue (left lane), followed by autoradiography (right lane; exposure time 24 h at −60 °C). Protein standards (kDa) were run in parallel.

(no-carrier-added, nca, 0.5 nmol of Na^{125}I with specific radioactivity >2000 Ci mmol^{-1}, 1 mCi) by means of CAT, high yield of radioiodination (48% for the lipid [^{125}I]-**12**) could be achieved only if the molar ratio NaI–CAT–Dcp substrate was raised to 1 : 10 : 100 (data not shown), similarly to radioiodination of tyrosine.[4d] Obviously, the use of peracetic acid should enhance the specific radioactivity of the [^{125}I]-Dcp-carrying probes near to the level of nca. However, the need of scrupulous manipulations with subnanomolar quantities of the Dcp substrate is the main obstacle here. The purification of radioiodinated probe from noniodinated precursor, complicates the procedure by necessity of HPLC (TLC does not provide separation), and therefore separation was not carried out.

Following the established procedure, PC probe [^{125}I]-**12** was incorporated into the outer leaflet of the FPV membrane (the molar ratio probe–FPV phosphatidylcholine, ~0.01),[18c] and photolysed. Then unbound lipids were removed by extraction with organic solvents, and the residue was analysed by PAGE, where the behaviour of viral proteins is well known.[18,19] As shown in Fig. 4, the only labelled protein was the membrane anchor HA_2. This pattern coincides completely with the established organisation of the viral envelope.[18]

The first application of the radioiodinated probe **12** for a study of mitochondrial ATP synthase ensemble in membrane gave new interesting data.[20]

It should be said in conclusion that the Dcp group, as shown in this study, is an efficient photoaffinity label. This, together with other advantages of the group, the convenience of introducing ^{125}I in one step just before use, the small size, and availability, make it a promising tool in biological studies.

Experimental

Absorption spectra were recorded with a LKB Ultrospec II 4050 spectrophotometer. ^{1}H NMR spectra were registered on a Bruker WM-500 spectrometer at 500 MHz and are reported in δ-units with tetramethylsilane as the internal standard. *J* values are given in Hz. Mass spectra were measured on a Varian MAT 44 apparatus at an ionisation potential of 70 eV, or a Finnigan MAT 9005 (ESI), or a MSVK instrument (^{252}Cf-plasma desorption tof, "Elektron" company, Sumy, Ukraine). Exact mass determinations were performed on a Q-tof (Micromass, Manchester, UK) system equipped with a nanospray interface. IR spectra were recorded on a Specord 751R spectrometer. Mps were measured on a Kofler hot-stage apparatus and are uncorrected. Elemental analysis were carried out only for the crystalline substances.

Analytical TLC was done on precoated plates (silica gel 60 F_{254}, RP-8_{254}, RP-18_{254}) from Merck. Column chromatography was carried out with Merck silica gel 60 (40–63 μm), RP-8 or RP-18 (25–40 μm); gel filtration—with Sephadex LH-20 from Pharmacia. HPLC was performed isocratically on an Altex 334 solvent delivery system equipped with a model 153 UV detector (254 nm). Slab PAGE was carried out on a home-made apparatus.

Photolyses were carried out in a Pyrex glass (2 mm walls) tube under stirring in an argon atmosphere using a medium pressure mercury lamp (30 W, emission max 365 nm) at the distance of 7 cm. To cut off any irradiation below 320 nm, the light was filtered through saturated aqueous $CuSO_4$ (1 cm layer).

Solvents were purified and dried by standard procedures; all evaporations were performed *in vacuo* at temperatures less than 40 °C. Dcp acid **1**,[8b] *p*-nitrophenyl trifluoroacetate,[21a] peracetic acid[4c] were synthesised as described earlier. Ester **2** was obtained by the treatment of acid **1** with freshly prepared and twice distilled into ether CH_2N_2. Lysophosphatidylcholine (LPC) was prepared from egg PC by phospholipase A_2 hydrolysis,[22] lyso-GM1—by hydrolysis of the natural ganglioside GM1.[23] Na^{125}I (100 mCi ml^{-1} in NaOH) was purchased from "Izotopy" (Sankt-Peterburg, Russia). All works with radioactivity were performed in a C-type laboratory. For quantitation of ^{125}I, a γ-counting system Compugamma 1282 from LKB Wallac was used. Autoradiographies were carried out with RX Fuji Medical X-ray films.

Fowl plague virus (influenza type A virus) was grown in chicken fibroblasts and purified as described earlier.[18c]

All manipulations with Dcp derivatives were carried out under yellow light.

Iodination of the Dcp group, and its photolysis

Methyl esters of 1-diazo-4-iodocyclopenta-2,4-dien-2-ylcarboxylic acid 3, 1-diazo-5-iodocyclopenta-2,4-dienyl-2-carboxylic acid 4 and 1-diazo-4,5-diiodocyclopenta-2,4-dien-2-ylcarboxylic acid 5. A suspension of chloroamine T trihydrate (2.49 g, 8.83 mmol) in methanol (7 ml) was added under stirring to a solution of ester **2** (112 mg, 0.73 mmol) and NaI (1.1 g, 7.3 mmol) in methanol (3 ml). In a few seconds, the resulting brown solution became yellow, and was stirred for 10–20 min extra. The solvent was evaporated partly *in vacuo* followed by filtration from the precipitate (iodoamine T and NaCl). The filtrate was desalted on a RP-8 column eluted with methanol–water, 4 : 6, then 1 : 1, 7 : 3, and pure methanol. A product of iodination (61 mg, ~30%), in aliquots, was further separated into individual substances by HPLC (7 μm silica SGX Tessek 4 × 250 mm column, 0.2% ether in hexane, 1 ml min^{-1}): the retention times for the products **3**, **4**, **5** are 32, 20, 15 min, respectively.

Photolysis of the ester 2 in cyclohexane. A solution of ester **2** in cyclohexane (6 mM) was irradiated (without $CuSO_4$ filter: $\lambda > 300$ nm) to yield compound **6a** (or **6b**); δ (C_6D_{12}) 6.63 (1 H, dt, $J_{3,4}$ or $J_{5,4}$ 5.38, $J_{3,5}$ or $J_{5,3}$ 1.47, 3-H or 5-H), 6.04 (1 H, dt, $J_{4,3}$ or $J_{4,5}$ 5.38, $J_{4,5}$ or $J_{4,3}$ 1.70, 4-H), 3.65 (3 H, s, Me), 3.55 (1 H, tt, $J_{ax,ax}$ 11.98, $J_{ax,eq}$ 3.25, cyclohexyl-H), 2.99 (2 H, t, $J_{5,4}$ or $J_{3,4}$ 1.47, 5-H or 3-H); δ(CD_3OD) 6.82 (1 H, dt, $J_{3,4}$ or $J_{5,4}$ 5.63, $J_{3,5}$ or $J_{5,3}$ 1.47, 3-H or 5-H), 6.42 (1 H, dt, $J_{4,3}$ or $J_{4,5}$ 5.63, $J_{4,5}$ or $J_{4,3}$ 1.47–1.70, 4-H), 3.97 (3 H, s, Me), 3.64 (1 H, tt, $J_{ax,ax}$ 11.67, $J_{ax,eq}$ 3.18, cyclohexyl-H), 3.38 (2 H, t, $J_{5,4}$ or $J_{3,4}$ 1.47, 5-H or 3-H), 1.50–1.63 (m, cyclohexyl); *m/z* (ESI) 207.2 (100%, M^+ + H), 229.2 (20, M^+ + Na); $C_{13}H_{18}O_2$ (M^+) requires 206.1307.

Photolyses of the iodinated esters 3, 4 and 5 in cyclohexane. The photolyses were performed at $\lambda > 320$ nm and yielded compounds **7a**: $\delta(C_6D_{12}$; assignments of resonances are given in accordance with the initial numeration of C-atoms in the diazocyclopentadiene ring) 6.89 (1 H, t, *J* 1.23, 3-H), 3.13 (2 H, d, *J* 1.22, 5-H); **7b**: 6.61 (1 H, dd, *J* 1.47, *J* 2.20, 5-H), 6.13 (1 H, dd, *J* 1.23, *J* 5.63, 3-H_{eq}), 6.05 (1 H, dd, *J* 2.20, *J* 5.63, 3-H_{ax}); **8a**: 6.95 (2 H, m, 3-H and 4-H), 5.08 (1 H, t, *J* 1.22, 5-H); **8b**: 6.91 (1 H, m, 3-H), 6.68 (1 H, dd, $J_{4,3}$ 2.20, $J_{4,1}$ 1.22, 4-H), 3.20 (1 H, m, 1-H). Multiplets in the spectra of the photolysed esters **3** and **4** at 3.63–3.65 ppm obviously are sets of methyl singlets, and the cyclohexyl 1-H resonance (tt) seems to be hidden under them. Compound **9**: $\delta(C_6D_{12})$ 6.57 (1 H, s, 3-H), 3.65–3.63 (1 H, m, presumably 1-H), 3.64 (3 H, s, Me). MS data (ESI) for the photolysed ester **3** (compounds **7a** + **7b** + **8a**): *m*/*z* 387.3 (100%, $M^+ + Na + MeOH$), 301.4 (40, $M^+ - OMe$); $C_{13}H_{17}IO_2$ (M^+) requires 332.0273; photolysed ester **4** (compounds **8a** + **8b** + **7b**): *m*/*z* 387.3 (100), 301.4 (50); photolysed ester **5** (compound **9**): *m*/*z* 456.8 (12, $M^+ - H$), 428.5 (18, $M^+ + H - OMe$); $C_{13}H_{16}I_2O_2$ (M^+) requires 457.9239.

Syntheses of phospholipid and ganglioside photoprobes

11-Hydroxyundecanoic acid. A solution of $NaNO_2$ (483 mg, 7 mmol) in H_2O (2 ml) was added to a solution of 11-aminoundecanoic acid (1 g, 5 mmol) in H_2O–AcOH, 4 : 1 (5 ml), then the mixture was heated at 100 °C for 1 h and allowed to cool to rt during several hours. After acidification with 1 M HCl to pH 3.0, the mixture was extracted with $CHCl_3$ (3 × 5 ml). The extract was evaporated, dried *in vacuo*, and the residue was subjected to silica gel column chromatography (gradient of a mixture *i*PrOH–AcOH, 10 : 1, in $CHCl_3$, 0⟶10%) to yield 11-hydroxyundecanoic acid (382 mg, 38%); mp 64–65 °C (from H_2O) (lit.,[21b] 65–66 °C); ν_{max}/cm^{-1} 3620 (OH), 2933 and 2860 (CH_2), 1700 (C=O), 1226 and 1200 (OH_{COOH}); $\delta(CDCl_3)$ 3.64 (2 H, t, HOCH_2), 2.35 (2 H, t, CH_2COOH), 1.64 (2 H, m, HOCH$_2$CH_2), 1.55 (2 H, m, CH_2CH$_2$COOH), 1.29 (12 H, m, $(CH_2)_6$).

Methyl 11-hydroxyundecanoate. AcCl (5 ml) was added under stirring to MeOH (50 ml). This mixture was added immediately to 11-hydroxyundecanoic acid (200 mg, 1 mmol). After 3.5 h at 40 °C, the reaction mixture was evaporated, and the residue was purified by silica gel column chromatography ($CHCl_3$–MeOH, 9 : 1) to give TLC pure oily product (115 mg, 53%).

Methyl 11-(trifluoromethylsulfonyloxy)undecanoate 10. A solution of methyl 11-hydroxyundecanoate (115 mg, 0.53 mmol) in dry $CHCl_3$ (6 ml) with pyridine (70 µl, 0.87 mmol) was added to a solution of Tf_2O (144 µl, 0.87 mmol) in $CHCl_3$ (6 ml) under stirring at −10 °C. After 2 h stirring at 0 °C, the reaction mixture was extracted with H_2O (3 × 3 ml), then the organic phase was filtered through cotton wool, evaporated and dried *in vacuo* to yield compound **10** (159 mg, 86%) as an individual colourless oil as indicated by TLC.

Methyl 11-(1-diazocyclopenta-2,4-dien-2-ylcarbonyloxy)-undecanoate 11. A solution of ester **10** (159 mg, 0.46 mmol) in dry acetone (2.6 ml) and triethylamine (80 µl) were added to a solution of acid **1** (20 mg, 0.15 mmol) in acetone (1 ml). The reaction mixture was stirred for 40 min at rt, then evaporated, and the residue was subjected to silica gel column chromatography (gradient of ethyl acetate in heptane, 0⟶7%) to yield TLC individual oily product **11** (25 mg, 50%); $\delta(CDCl_3)$ 7.02 (1 H, dd, $J_{5',4'}$ 4.64, $J_{5',3'}$ 1.96, 5′-H), 6.82 (1 H, dd, $J_{3',4'}$ 3.18, $J_{3',5'}$ 1.96, 3′-H), 6.07 (1 H, dd, $J_{4',5'}$ 4.60, $J_{4',3'}$ 3.20, 4′-H), 4.24 (2 H, t, *J* 6.72, DcpOCH_2), 3.66 (3 H, s, Me), 1.70 (2 H, t, *J* 6.72, CH_2COO), 1.61 (2 H, m), 1.38 (2 H, m), 1.29 (12 H, m, $(CH_2)_6$); λ_{max} (EtOH)/nm 215 (ε/dm^3 mol^{-1} cm^{-1} 12400) and 314 (15900); *m*/*z* (^{252}Cf) 334 (100%, M^+); $C_{18}H_{26}N_2O_4$ (M^+) requires 334.1932.

1-*O*-Acyl-2-*O*-[11-(1-diazocyclopenta-2,4-dien-2-yl-2-carbonyloxy)undecanoyl]-*sn*-glycero-3-phosphocholine 12. Aqueous KOH (5%, 0.4 ml) was added to a solution of methyl ester **11** (25 mg, 75 µmol) in propan-2-ol (16 ml), and the mixture was incubated at rt (about 14 h) till trace amounts of the DcpOH **1** appeared (TLC control). After neutralisation with 1 M HCl and evaporation, the residue was suspended in H_2O (1 ml), acidified with 1 M HCl to pH 2–3 and extracted with $CHCl_3$ (3 × 0.5 ml). The extracted material was subjected to silica gel column chromatography (gradient of a mixture *i*PrOH–AcOH, 10 : 1, in $CHCl_3$, 0⟶5%), then on a RP-18 column (MeOH–H_2O, 7 : 3, then 9 : 1) to yield the TLC pure 11-(Dcp-oxy)undecanoic acid (8.2 mg, 34%); UV spectrum is similar to that of the methyl ester **11**.

The LPC (5 mg, 10 µmol) was converted to the trifluoroacetate form by co-evaporation with trifluoroacetic acid (5 µl) in $CHCl_3$ (0.5 ml). After drying overnight under high vaccum, the resulting LPC salt and 11-(Dcp-oxy)undecanoic acid (3.2 mg, 10 µmol) were dissolved in dry $CHCl_3$ (0.4 ml), a suspension of 4-aminopyridine (5 mg, 53 µmol) in $CHCl_3$ (0.6 ml), and DCC (5 mg, 25 µmol) in CCl_4 (25 µl) were added, and the mixture was stirred for 24 h at rt under an argon atmosphere. After filtration and concentration *in vacuo*, the reaction mixture was purified by gel filtration (1 × 20 cm column) in a mixture $CHCl_3$–MeOH, 1 : 1, followed by chromatography on a RP-18 column (MeOH–$CHCl_3$–H_2O, 10 : 1 : 1, then 10 : 1 : 0.25) to yield pure (TLC) phospholipid **12** (3.5 mg, 44%); $\delta(CDCl_3)$ 7.03 (1 H, br dd, $J_{5',4'}$ 4.57, $J_{5',3'}$ 1.83, 5′-H), 6.82 (1 H, br t, 3′-H), 6.07 (1 H, br dd, $J_{4',5'}$ 4.27, $J_{4',3'}$ 3.35, 4′-H), 5.21 (1 H, m), 4.38–4.52 (4 H, br m), 4.24 (2 H, t, *J* 6.71, DcpOCH_2), 3.92–4.17 (4 H, br m), 3.42 (9 H, s), 2.26–2.36 (4 H, m), 1.64–1.78 (6 H, m), 1.22–1.36 (~36 H, m), 0.89 (3 H, t, *J* 6.71, Me); *m*/*z* (ESI) 798.504 (100%, $M_1^+ + H$), 820.519 (34, $M_1^+ + Na$), 836.477 (58, $M_1^+ + K$); $C_{41}H_{72}N_3PO_{10}$ (M_1^+) requires 797.5015 [($M_1^+ + H$) requires 798.5093]; 826.546 (70, $M_2^+ + H$), 848.545 (15, $M_2^+ + Na$), 864.515 (30, $M_2^+ + K$); $C_{43}H_{76}N_3PO_{10}$ ($M_2^+ = M_1^+ + 2CH_2$) requires 825.5328 [($M_2^+ + H$) requires 826.5406].

***p*-Nitrophenyl 11-(1-diazocyclopenta-2,4-dien-2-ylcarbonyloxy)undecanoate 13.** A solution of *p*-nitrophenyl trifluoroacetate (5.8 mg, 24 µmol) in dry pyridine (200 µl) was added to the 11-(Dcp-oxy)undecanoic acid (4 mg, 12 µmol) and stirred for 5 h at rt. After evaporation, the reaction mixture was purified by silica gel column chromatography (gradient of ethyl acetate in toluene, 0⟶2%) to yield yellow oily product **13** (2.7 mg, 50%); *m*/*z* (^{252}Cf) 441.6 (60%, M^+); $C_{23}H_{27}N_3O_6$ (M^+) requires 441.1960.

***p*-Nitrophenyl iodoacetate 14.** The solutions of *p*-nitrophenol (46 mg, 0.36 mmol) in dry $CHCl_3$ (3 ml) and DCC (80 mg, 0.4 mmol) in CCl_4 (0.4 ml) were added at 0 °C under stirring to a solution of iodoacetic acid (56 mg, 0.3 mmol) in $CHCl_3$ (3 ml). After 1 h stirring, the reaction mixture was allowed to stand at rt overnight. Then it was filtered through cotton wool, evaporated, redissolved in toluene, filtered once again and evaporated. The residue was purified by silica gel column chromatography in toluene with 0.5% AcOH to yield product **14** (51 mg, 55%); mp 80 °C (from EtOH); $\delta(CDCl_3)$ 8.30 (2 H, d, $J_{5',6'} = J_{3',2'}$ 9.17, 3′-H and 5′-H), 7.33 (2 H, d, $J_{2',3'} = J_{6',5'}$ 9.17, 2′-H and 6′-H), 3.95 (2 H, s, ICH_2CO) (Found: C, 31.24; H, 2.00; N, 4.42; I, 41.00. $C_8H_6NIO_4$ requires C, 31.29; H, 1.97; N, 4.56; I, 41.33%).

***p*-Nitrophenyl (1-diazocyclopenta-2,4-dien-2-ylcarbonyloxy)-acetate 15.** Triethylamine (28 µl, 200 µmol) was added under stirring to a solution of Dcp-OH acid **1** (13.8 mg, 0.1 mmol) in dry acetone (1 ml), followed by the addition of ester **14** (31.5 mg, 0.1 mmol) in acetone (1 ml). The reaction mixture was

stirred overnight at rt. After evaporation, the residue was purified by RP-18 column chromatography (MeOH–H_2O, 1 : 1, then 6 : 4) to yield product **15** (25 mg, 80%); δ($CDCl_3$) 8.30 (2 H, d, $J_{5',6'} = J_{3',2'}$ 9.15, 3′-H and 5′-H), 7.36 (2 H, d, $J_{2',3'} = J_{6',5'}$ 9.15, 2′-H and 6′-H), 7.12 (1 H, dd, $J_{5'',4''}$ 4.58, $J_{5'',3''}$ 1.98, 5″-H Dcp), 6.97 (1 H, dd, $J_{3'',4''}$ 3.10, $J_{3'',5''}$ 2.14, 3″-H Dcp), 6.14 (1 H, dd, $J_{4'',5''}$ 4.58, $J_{4'',3''}$ 3.30, 4″-H Dcp), 5.04 (2 H, s, OCH_2COO); *m*/*z* (^{252}Cf) 315.7 (80%, M^+); $C_{14}H_9N_3O_6$ (M^+) requires 315.0551.

11-(1-Diazocyclopenta-2,4-dien-2-ylcarbonyloxy)undecanoyl GM1 16. Triethylamine (1 µl, 7 µmol) and a solution of *p*-nitrophenyl ester of **13** (0.53 mg, 1.2 µmol) in DMSO (50 µl) were mixed with a solution of lyso-GM1 (1 mg, ~1 µmol) in DMSO (50 µl). After 16 h incubation at rt, a drop of water, and $CHCl_3$–MeOH, 1 : 1 (0.1 ml) were added to the reaction mixture; the following gel filtration (0.5 × 20 cm column) in a mixture $CHCl_3$–MeOH, 1 : 1, yielded TLC-pure ganglioside **16** (~98% on the basis of UV absorption); UV spectrum is similar to that of the ester **11**; δ(CD_3OD) (data for the several protons) 7.35 (1 H, br dd, $J_{5',4'}$ 4.58, $J_{5',3'}$ 1.83, 5′-H), 6.97 (1 H, br t (dd, $J_{3',4'}$ 3.4, $J_{3',5'}$ 1.8), 3′-H), 6.26 (1 H, br dd, $J_{4',5'}$ 4.37, $J_{4',3'}$ 3.46, 4′-H), 5.87 (1 H, dt, $J_{5,4}$ 15.34, $J_{5,6}$ 7.72, 5-H of (*E*)C=C), 5.64 (1 H, dd, $J_{4,5}$ 15.21, $J_{4,3}$ 7.78, 4-H of (*E*)C=C), 4.63, 4.55, and 4.49 (3 × 1 H, 3 dd, *J* 7.61, 8.03, and 7.81, 1-H hexapyranoses β), 4.05 (1 H, m, 2-H), 2.92 (1 H, dd, ^2J 12.20, ^3J 4.42, 3-H_{eq} NeuAcα), 2.36 (2 H, t, *J* 7.52, CH_2CO), 2.20 and 2.18 (2 × 3 H, 2 s, 2 NAc), 2.11 (1 H, dd ~ t, *J* 12.10, 3-H_{ax} NeuAcα), 1.91 (2 H, m, CH_2CH_2CO), 1.45–1.55 (~36 H, m), 1.08 (3 H, t, *J* 7.10, Me); *m*/*z* (ESI) 1582.875 (100%, M_1^+ + H), 1604.869 (33, M_1^+ + Na), 1620.837 (61, M_1^+ + K); $C_{72}H_{119}N_5O_{33}$ (M_1^+) requires 1581.7885 [(M_1^+ + H) requires 1582.7963]; 1610.912 (39, M_2^+ + H), 1632.893 (25, M_2^+ + Na), 1648.866 (49, M_2^+ + K); $C_{74}H_{123}N_5O_{33}$ ($M_2^+ = M_1^+ + 2CH_2$) requires 1609.8198 [(M_2^+ + H) requires 1610.8276].

(1-Diazocyclopenta-2,4-dien-2-ylcarbonyloxy)acetyl GM1 17. Ganglioside **17** was synthesised as described for the probe **16**, from lyso-GM1 (1 µmol) and *p*-nitrophenyl Dcp-oxyacetate **15** (380 µg, 1.2 µmol); yield 88% (on the basis of the UV absorption); δ(CD_3OD) 7.40 (1 H, br dd, $J_{5',4'}$ 4.6, $J_{5',3'}$ 1.8, 5′-H), 7.09 (1 H, br t, 3′-H), 6.29 (1 H, br t (dd, $J_{4',5'}$ 4.3, $J_{4',3'}$ 3.3), 4′-H), 5.89 (1 H, dt, $J_{5,4}$ 15.32, $J_{5,6}$ 7.70, 5-H of (*E*)C=C), 5.65 (1 H, dd, $J_{4,5}$ 15.20, $J_{4,3}$ 7.72, 4-H of (*E*)C=C), 4.63, 4.59, and 4.50 (3 × 1 H, 3 dd, *J* 7.60, 8.02, and 8.02, 1-H hexapyranoses β), 4.03 (1 H, m, 2-H), 2.92 (1 H, dd, ^2J 12.20, ^3J 4.40, 3-H_{eq} NeuAcα), 2.85 (2 H, s, $COCH_2ODcp$), 2.20 (2 × 3 H, br s, 2 NAc), 1.45–1.57 (~22 H, m), 1.09 (3 H, t, *J* 7.10, Me); *m*/*z* (ESI) 1456.740 (10%, M_1^+ + H), 1478.714 (87, M_1^+ + Na), 1494.701 (90, M_1^+ + K); $C_{63}H_{101}N_5O_{33}$ (M_1^+) requires 1455.6477 [(M_1^+ + H) requires 1456.6555]; 1484.723 (20, M_2^+ + H), 1506.740 (67, M_2^+ + Na), 1522.722 (100, M_2^+ + K); $C_{65}H_{105}N_5O_{33}$ ($M_2^+ = M_1^+ + 2CH_2$) requires 1483.6790 [(M_2^+ + H) requires 1484.6868].

PAL of the membrane proteins of the influenza virus

Radioiodinated phospholipid probe [^{125}I]-12. A solution of the phosphatidylcholine **12** (~1.6 µg, ~2 nmol) in 10 µl of AcOH was added to a solution of Na^{125}I (nca; 0.5 nmol, >2000 Ci mmol^{-1}, 1 mCi) in aqueous NaOH (10 µl, pH 10.0) in commercial ampoule, followed by addition of ~11% peracetic acid solution in AcOH (5 µl). After short vortexing and 5 min incubation at rt, the reaction was quenched with 10% $NaHSO_3$ (50 µl) and 5 M NaOH (35 µl). Then the mixture was extracted with a $CHCl_3$–MeOH mixture (2 : 1, 50 µl × 2) by vortexing. The organic phases were collected and co-evaporated, the residue was dissolved in an MeOH–$CHCl_3$–H_2O system, 10 : 1 : 1, applied on an RP-18 micro-column (200 µl) and washed with MeOH–H_2O (9 : 1, 200 µl, then 3 : 2, 1 ml). Elution with MeOH (2 ml) yielded ~0.96 mCi of the probe [^{125}I]-**12** (95% of radiochemical purity, TLC). After concentration, the residue was dissolved in $CHCl_3$ (1 ml), stored at −20 °C and used for PAL the next day. (All manipulations with methanolic solutions were performed rapidly to avoid perceptible hydrolysis of ester bonds in nmol of [^{125}I]-**12**.)

Photolysis of [^{125}I]-12 in the fowl plague virus. A solution of the probe [^{125}I]-**12** (80 µCi) in ethanol (10 µl) was added under argon to the virus suspensions in phosphate buffered saline, pH 7.0 (0.1 mg protein ml^{-1}, 1 ml, pre-bubbled with argon) and incubated for 3 h at 37 °C and gentle rocking. Then the suspensions were irradiated for 5 min at $\lambda > 320$ nm ($CuSO_4$ filter) and ambient temperature, with stirring. After delipidation of the samples with 3 volumes of $CHCl_3$–MeOH, 1 : 3, v/v (sodium dodecyl sulfate was added up to 0.4% to some samples, and they were incubated for 15 min prior to the addition of organic solvents to achieve more exhaustive delipidation), protein precipitates (2000*g* centrifugation) were dried *in vacuo*, and then subjected to PAGE according to the established procedure,[18c,19] with drying and autoradiography.

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