162. Synthesis of New Phospholipids Linked to Steroid-Hormone Derivatives Designed for Two-Dimensional Crystallization of Proteins

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The synthesis of phospholipids $\mathbf{1}_n$–$\mathbf{3}_n$, rationally designed for two-dimensional crystallization of progesterone and estradiol receptors, is reported. The structure of these lipids provides them with essential properties such as fluidity and stability when spread into monolayers at the air/H_2O interface, affinity for the protein to be crystallized, and accessibility of the ligand under the lipid monolayer.

Introduction. – For a better understanding of enzymatic mechanisms and biological activities, knowledge of the three-dimensional (3D) structures of the macromolecules involved in these processes could be of value. Structural approaches using X-ray crystallography or nuclear magnetic resonance (NMR) need several milligrams (10–100 mg) of the highly purified protein (up to 95–98% pure). In the last ten years, a combination of two-dimensional (2D) crystallization and electron-microscopy techniques led to structure elucidation of proteins with resolution comparable to those reached by X-ray crystallography [1–14]. This new approach requires only a few micrograms of partially purified biological material and allows the study of very high molecular weight proteins, whether they are soluble or not.

2D Crystallization of proteins can be induced using lipid layers. The lipid, whose polar head is attached to a specific ligand of the protein, is allowed to spread at the air/H_2O interface of a microvolume (typically 15 µl) of a buffered solution of the protein (*Figs. 1a* and *1b*). Molecular recognition of the ligand by the protein results in placing the macromolecules in a plane with an orientation identical to that of its neighbours, except by rotation (axis perpendicular to the plane; *Fig. 1c*). Due to the properties of the phospholipid (monolayer fluidity and stability) and those of the aqueous phase (ionic strength, pH, nature of ions in solution), protein-lipid complexes interacting one on each other (electrostatic interactions, H-bonds, hydrophobic insertions) move in rotation and translation to form 2D arrays of the protein (*Fig. 1d*). These protein arrays are studied by electron microscopy, and after image processing, the 3D structure of the macromolecule can be obtained.

In order to study the structure of steroid-hormone binding receptors, we synthesized molecules $\mathbf{1}_n$–$\mathbf{3}_n$ whose structure confer to them specific physical and biological characteristics (*Fig. 2*). One of the most important points in the approach is the fluidity of the lipid film. Till now, all successfully performed experiments show that 2D crystals can be

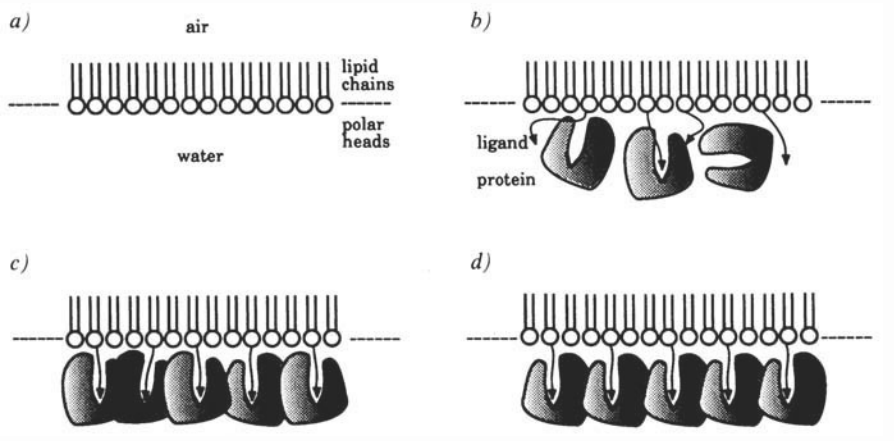


Fig. 1. *Principle of the two-dimensional crystallization technique of biological macromolecules onto a lipid monolayer at the air/water interface.* For comments, see text.

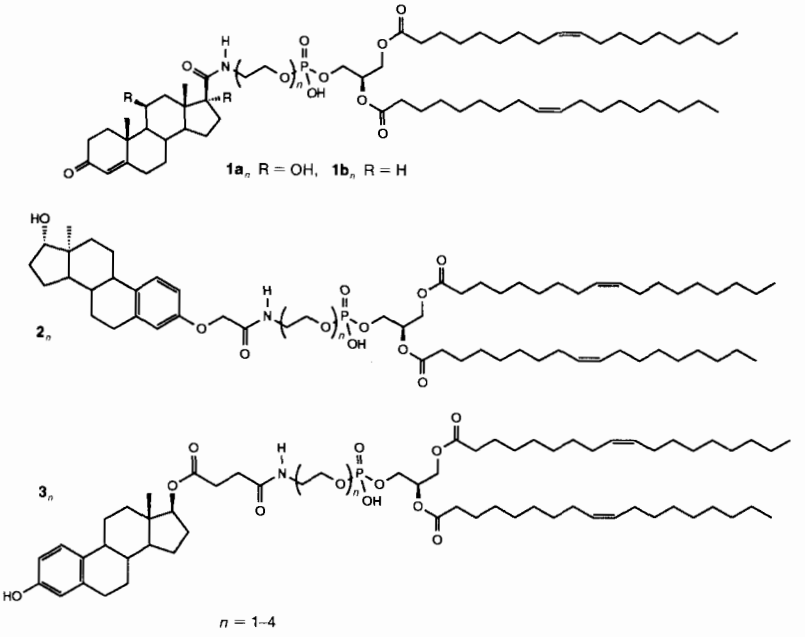


Fig. 2. *Amphiphilic structures especially designed for two-dimensional crystallization experiments with hormone-binding receptors*

obtained only with a lipid layer in a fluid phase. This can be easily interpreted considering that when the lipid monolayer is in a crystalline phase, the lipid cohesion is too high to provide enough mobility to the lipid-protein complexes and subsequently strongly impedes rotational and translational motions that lead to crystalline arrays of protein molecules. As determined by a previous study of ours [15], lipid chains should be at least sixteen C-atoms long to form stable enough films and should contain a mid-chain (*Z*)-configurated unsaturation. This last point is essential to provide the lipid layer with the adequate fluidity at room temperature and at the surface pressure at which the crystallization experiments are performed (30–40 mN/m) [16–18].

Another very important point to get 2D crystals is the necessity to reach a high concentration of the proteins in the plane, so that they are close together enough to interact and crystallize. This local protein concentration is directly related to two independent factors: *i*) the affinity of the protein for the ligand (the higher it is, the better the overall system should work) and *ii*) the accessibility of the recognition site of the protein to the ligand. Affinity depends on the nature of the chemically modified ligand. Accessibility is dependent on the distance between the biologically active moiety and the phosphate group. The crucial importance of the length of the spacer was shown in a study of a very special enzyme, DNA gyrase [14]. A too short (*Figs. 3a* and *3b*) as well as a too long

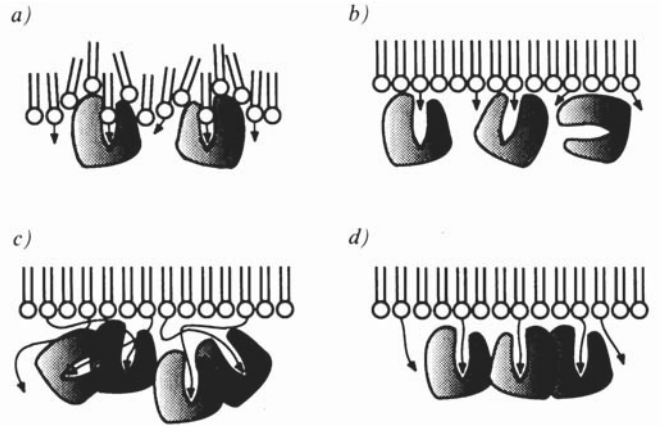


Fig. 3. *Influence of spacer length on 2D crystallization* (model considerations): a) b) *spacer too short;* c) *spacer too long;* d) *spacer of optimum length spacer*

distance (*Fig. 3c*) are unfavourable to the obtention of 2D proteins crystals. The optimum size for the spacer (*Fig. 3d*) is of course unpredictable as long as the 3D structure of the protein (or at least the geometrical configuration of the active site) is not known. So, it is useful to have lipids of different spacer lengths available for crystallization experiments. A way to modulate lateral accessibility of the ligand would be the dilution of the lipid bound to the ligand with lipid devoid of the ligand (*Fig. 4*). Theoretically, this would allow a more ordered state of the proteins at the interface to evolve. However, results presented throughout the literature [3–14] do not allow any clear interpretation at the

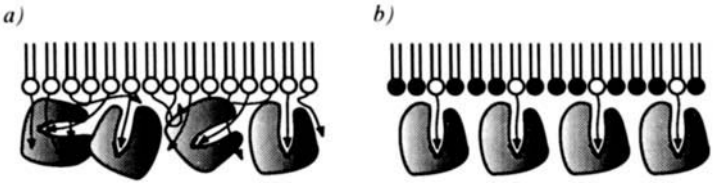


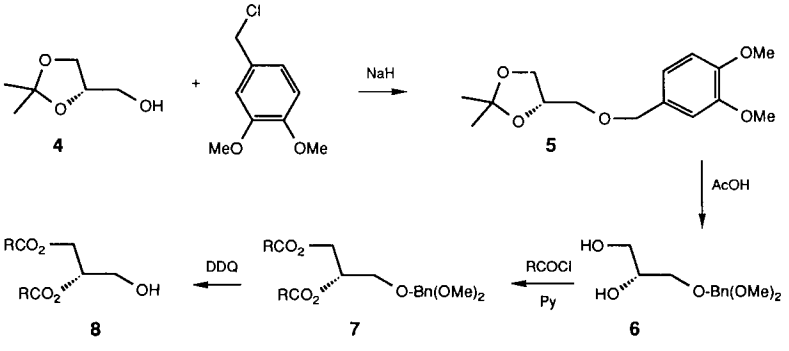
Fig. 4. *Lateral accessibility modified by dilution of the lipid linked to the ligand* (white polar head) *with a nonbinding lipid* (black polar head). For comments, see text.

moment, and lateral accessibility does not seem to be a key factor for getting 2D crystals of proteins.

Here, we focussed our interest on the progesterone and estradiol receptors that are involved in a number of biological processes [19–21]. Hormonal receptors are present in quite tiny amounts, and until very recently, cloned, overexpressed, and purified proteins were not available [22]. Taking in account these difficulties, 2D crystallization still remains the method of choice for getting 3D structures, although at limited resolution, of hormonal receptors in general, progesterone and estradiol receptors in particular. We have already described the synthesis and physical study of some phospholipids linked to steroid-hormone derivatives [23] [15]. The new type of lipid structures, whose synthesis is described herein, allows a rational approach to the 2D-crystallization technique as applied to the study of progesterone and estradiol receptors, examining systematically a number of parameters such as the length of the spacer, the way the steroid is linked to the lipid, and the nature of the medium-distance environment of the ligand.

Chemistry. – The synthetic scheme is based on a convergent approach proceeding through the preparation of the three intermediates **8** (*Scheme 1*), **13**$_n$ (*Scheme 2*), and a steroid derivative (*e.g.* **17**, *Scheme 3*) that are coupled together to lead to the final structures **1**$_n$–**3**$_n$ (*Fig. 2*). The lipid skeleton of **1**$_n$–**3**$_n$ is elaborated using (*S*)-2,2-dimethyl-1,3-dioxolane-4-methanol (**4**), readily obtained from D-mannitol [24] (*Scheme 1*). The presence of two unsaturated bonds in the target diacyl-glycerol moiety **8** introduces some complication in the synthesis that was not, up to now, satisfactorily solved. The use of a 3,4-dimethoxybenzylic ether [25] [26] as protective group for the primary alcohol **4**(→**5**)

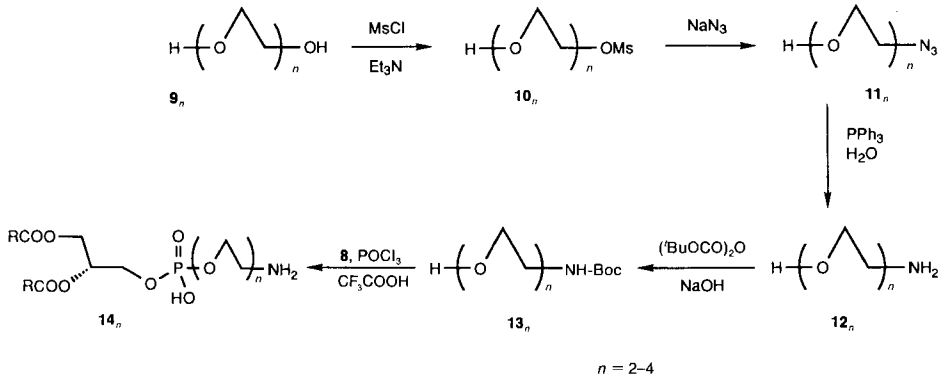
Scheme 1



allows now to overcome the general difficulties encountered in the synthesis of this type of compounds. After hydrolysis (→ **6**) and acylation (→ **7**), **8** is obtained with very good yields.

The hydrophilic spacer[1]) introduced between the phosphate group and the ligand is obtained from di-, tri-, and tetraethylene glycols $\mathbf{9}_n$ (*Scheme 2*). The general route proceeds through mono-mesylation (→ $\mathbf{10}_n$), followed by a nucleophilic displacement with sodium azide (→ $\mathbf{11}_n$). The general displacement procedures employing DMSO as solvent are not suitable in our case, the hydrophilic character of the molecules leading to much difficulties on purification. The use of MeCN appears to be more efficient and gives much higher yields. Subsequent reduction of the azides $\mathbf{11}_n$ with PPh_3/H_2O leads to the amines $\mathbf{12}_n$ that are further protected as *tert*-butyl carbamates $\mathbf{13}_n$ (n = 2–4). This step is essential to avoid polymerization during coupling with $POCl_3$ and diacyl-glycerol **8** yielding $\mathbf{14}_n$ (n = 2–4; *Scheme 2*). In the case of the shortest spacer 2-aminoethanol ($\mathbf{12}_1$), this protection is not necessary, since the reaction with **8** in $POCl_3$/HCl proceeds *via* a cyclic chlorophosphoramidate intermediate.

Scheme 2



Finally, 3-oxoandrost-4-ene-17β-carboxylic acid and 11β,17α-dihydroxy-3-oxoandrost-4-ene-17β-carboxylic acid, obtained by periodic-acid oxidation of deoxycorticosterone and cortisol [27] [28], [(17β-hydroxyestra-1,3,5(10)-trien-3-yl)oxy]acetic acid (**17**) and estra-1,3,5(10)-triene-3,17β-diol 17β-hemisuccinate [29], obtained from estra-1,3,5(10)-triene-3,17β-diol (**16**; *Scheme 3*), are coupled with the different deprotected phospholipids $\mathbf{14}_n$ through an amide junction, using DCC with 4-(dimethylamio)pyridine or benzotriazol-1-ol (HOBt), to give compounds $\mathbf{1a}_n$, $\mathbf{1b}_n$, $\mathbf{2}_n$, and $\mathbf{3}_n$, respectively. The presence of numerous different functionalities on the two moieties to be coupled requires very precise reaction conditions in order to achieve satisfactory yields. The work-out of these conditions was essential to facilitate the further purifications that are especially difficult with this type of amphiphilic compounds.

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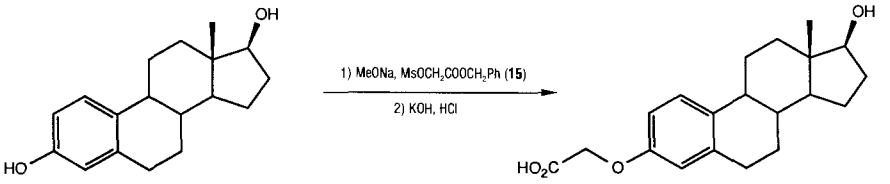
[1]) An additional problem encountered with steroid-hormone receptors related to the technique is the hydrophobicity of the ligands. These must be drawn far enough into the aqueous phase through the presence of the highly hydrophilic spacer.

Scheme 3



16 **17**

Further work to study the mechanical behaviour of these compounds spread into a monolayer at the air/H_2O interface as well as their biological potential for 2D crystallization of progesterone and estradiol receptors are in progress.

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Experimental Part

General. Tetrahydrofuran (THF) was distilled over Na/benzophenone and CH_2Cl_2 over CaH_2, just before use. Reactions were monitored by TLC (*Merck* precoated plates 0.25 mm, silica gel *60*, F_{254}); solvent ratios as *v/v*. Products were purified by column chromatography (CC; *Merck* silica gel *60*, 0.040–0.063 mm, 230–400 mesh ASTM). ^{1}H- and ^{13}C-NMR spectra: *Bruker-WP-200-Sy* spectrometer; chemical shifts δ in ppm rel. to an internal reference (tetramethylsilane at 0.00 ppm, $CHCl_3$ at 7.27 ppm, or MeOH at 3.35 ppm; the latter for $CDCl_3/CD_3OD$ solns.), *J* in Hz. MS (negative-ion mode): anal. *VG-ZAB-HF* double-focussing instrument (mass range: 3 kda at 8 keV ion energy), fitted with a Xe-atom gun; mTEA, triethanolamine matrix; mNBA-TSA, nitrobenzyl-alcohol/4-toluenesulfonic-acid matrix.

(S)-4-[(3,4-Dimethoxybenzyloxy)methyl]-2,2-dimethyl-1,3-dioxolane (**5**). NaH (2.7 g, 113.5 mmol, 2 equiv.) was added by portions to (*S*)-2,2-dimethyl-1,3-dioxolane-4-methanol [24] (**4**; 7.5 g, 1 equiv.) and hexamethylphosphoric triamide (HMPA; 1 ml, 0.1 equiv.) in anh. THF (250 ml) at 0°. The mixture was stirred for 10 min, then 3,4-dimethoxybenzyl chloride (10.6 g, 1 equiv.) was added and refluxed for 12 h. Excess NaH was decomposed with H_2O (50 ml) and hexane (100 ml). The aq. phase was washed twice with Et_2O (25 ml), the combined org. phase dried (Na_2SO_4) and evaporated, and the residue chromatographed (silica gel, Et_2O/hexane 1:1): **5** as colourless liquid (13.4 g, 84%). TLC: R_f 0.4 (Et_2O/hexane 1:1); R_f 0.6 (AcOEt). ^{1}H-NMR ($CDCl_3$, 200 MHz): 6.90 (*s*, 1 H); 6.85 (*m*, 2 H); 4.54, 4.48 (*AB*, J_{AB} = 10.4, 2 H); 4.30 (*m*, 1 H); 4.05 (*dd*, *J* = 8.2, 6.3, 1 H); 3.90 (*s*, 3 H); 3.88 (*s*, 3 H); 3.73 (*dd*, *J* = 8.2, 6.3, 1 H); 3.54 (*dd*, *J* = 9.8, 5.7, 1 H); 3.45 (*dd*, *J* = 9.8, 5.5, 1 H); 1.42 (*s*, 3 H); 1.37 (*s*, 3 H). Anal. calc. for $C_{15}H_{22}O_5$: C 63.83, H 7.80; found: C 63.71, H 7.63.

(R)-3-(3,4-Dimethoxybenzyloxy)propane-1,2-diol (**6**). Dioxolane **5** (12.3 g, 43.5 mmol, 1 equiv.) in 10% AcOH/H_2O (40 ml) was heated at 70–80° for 2 h. The mixture was lyophilized and chromatographed (AcOEt): **6** as colourless liquid (9.9 g, 94%). TLC (AcOEt): R_f 0.3. ^{1}H-NMR ($CDCl_3$, 200 MHz): 6.85 (*m*, 3 H); 4.49 (*s*, 2 H); 3.95–3.81 (*m*, 1 H); 3.89 (*s*, 3 H); 3.88 (*s*, 3 H); 3,71 (*dd*, *J* = 11.5, 3.8, 1 H); 3.62 (*dd*, *J* = 11.5, 6.9, 1 H); 3.57 (*dd*, *J* = 9.6, 1.8, 1 H); 3.50 (*dd*, *J* = 9.6, 3.8, 1 H). Anal. calc. for $C_{12}H_{18}O_5$: C 59.50, H 7.44; found: C 59.46, H 7.39.

(S)-3-(3,4-Dimethoxybenzyloxy)propane-1,2-diyl Dioleate (**7**). Oleoyl chloride (22.5 ml, 68.2 mmol, 2 equiv.) was added dropwise at r.t. to **6** (6.2 g. 1 equiv.) and pyridine (6.1 ml, 2.2 equiv.) in CH_2Cl_2 (200 ml). The mixture was refluxed overnight. After addition of Et_2O (100 ml), the precipitate was filtered and the filtrate washed with 5% HCl soln., aq. $NaHCO_3$ soln., and brine. The org. phase was dried and evaporated and the residue chromatographed (Et_2O/hexane 3:7): **7** as colourless oil (24.5 g, 94%). TLC (Et_2O/hexane 3:7): R_f 0.5. ^{1}H-NMR ($CDCl_3$, 200 MHz): 6.86 (*m*, 3 H); 5.35 (*td*, *J* = 4.5, 1.8, 4 H); 5.26 (*m*, 1 H); 4.49, 4.46 (*AB*, J_{AB} = 11.7, 2 H); 4.35 (*dd*, *J* = 11.9, 3.8, 1 H); 4.18 (*dd*, *J* = 11.9, 6.4, 1 H); 3.90 (*s*, 3 H); 3.88 (*s*, 3 H); 3.57 (*d*, *J* = 5.2, 2 H); 2.30 (*td*, *J* = 15.4, 7.4, 4 H); 2.22–1.91 (*m*, 8 H); 1.60–1.40 (*m*, 4 H); 1.42–1.15 (*m*, 40 H); 0.88 (*t*, *J* = 6.1, 6 H).

(S)-3-Hydroxypropane-1,2-diyl Dioleate (**8**). To **7** (11.3 g, 1 equiv.) in CH_2Cl_2/H_2O 18:1 (30 ml), 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (= 4,5-dichloro-3,6-dioxocyclohexa-1,4-diene-1,2-dicarbonitrile; 3.33 g, 14.6 mmol, 1 equiv.) was added. The soln. was allowed to react for 2 h, then it was filtered, decanted, dried, and evaporated. CC (Et_2O/hexane 4:6) yielded **8** [30] as slightly yellow oil (7.6 g, 84%). TLC (Et_2O/hexane 1:1). R_f 0.4. ^{1}H-NMR ($CDCl_3$, 200 MHz): 5.40–5.32 (*m*, 4 H); 5.09 (*q*, J = 5.1, 1 H); 4.33 (*dd*, J = 12.3, 4.6, 1 H); 4.23 (*dd*, J = 11.9, 5.5, 1 H); 3.74 (*d*, J = 5.0, 2 H); 2.34 (*td*, J = 7.4, 3.2, 4 H); 2.12–1.92 (*m*, 8 H); 1.75–1.55 (*m*, 4 H); 1.45–1.15 (*m*, 40 H); 0.89 (*t*, J = 4.4, 3 H).

2-{2-[(Methylsulfonyl)oxy]ethoxy}ethanol (**10**$_2$). Methanesulfonyl chloride (7.13 ml, 94.3 mmol, 1 equiv.) was added dropwise to diethylene glycol **9**$_2$ (10 g, 1 equiv.) and Et_3N (13.2 ml, 1 equiv.) in dry THF (100 ml) at −5°. After 2 h, the soln. was allowed to warm to r.t. and stirred overnight. The precipitate was filtered, the filtrate evaporated, and the residue chromatographed (AcOEt/hexane 8:2 to 1:0): **10**$_2$ as colourless liquid (7.8 g, 45%). TLC (AcOEt): R_f 0.5. ^{1}H-NMR ($CDCl_3$, 200 MHz): 4.39–4.36 (*m*, 2 H); 3.84–3.71 (*m*, 4 H); 3.66–3.59 (*m*, 2 H); 3.06 (*s*, 3 H). Anal. calc. for $C_5H_{12}O_5S$: C 32.61, H 6.52; found: C 32.48, H 6.55.

Compounds **10**$_3$ and **10**$_4$ were prepared following the same procedure.

2-{2-{2-[(Methylsulfonyl)oxy]ethoxy}ethoxy}ethanol (**10**$_3$): Yield 45%. ^{1}H-NMR ($CDCl_3$, 200 MHz): 4.40–4.35 (*m*, 2 H); 3.79–3.67 (*m*, 8 H); 3.62–3.58 (*m*, 2 H); 3.07 (*s*, 3 H). Anal. calc. for $C_7H_{16}O_6S$: C 36.84, H 7.02; found: C 36.86, H 6.94.

2-{2-{2-{2-[(Methylsulfonyl)oxy]ethoxy}ethoxy}ethoxy}ethanol (**10**$_4$): Yield 44%. ^{1}H-NMR ($CDCl_3$, 200 MHz): 4.40–4.35 (*m*, 2 H); 3.78–3.57 (*m*, 14 H); 3.07 (*s*, 3 H). Anal. calc. for $C_9H_{20}O_7S$: C 39.70, H 7.35; found: C 39.45, H 7.23.

2-(2-Azidoethoxy)ethanol (**11**$_2$). For 36 h, **10**$_2$ (3.1 g, 29 mmol, 1 equiv.) was refluxed with NaN_3 (2.8 g, 1.5 equiv.) in dry MeCN (50 ml). H_2O (20 ml) was added and the mixture extracted with CH_2Cl_2. After drying and evaporation, the residue was chromatographed (AcOEt): **11**$_2$ as colourless liquid (3.5 g, 92%). TLC (AcOEt): R_f 0.5. ^{1}H-NMR ($CDCl_3$, 200 MHz): 3.64–3.50 (*m*, 6 H); 3.32 (*t*, J = 4.2, 2 H). Anal. calc. for $C_4H_9N_3O_2$: C 36.64, H 6.87; found: C 36.53, H 6.80.

Compounds **11**$_3$ and **11**$_4$ were obtained in the same way.

2-[2-(2-Azidoethoxy)ethoxy]ethanol (**11**$_3$): Yield 88%. ^{1}H-NMR ($CDCl_3$, 200 MHz): 3.71–3.52 (*m*, 10 H); 3.35 (*t*, J = 5.0, 2 H). Anal. calc. for $C_6H_{13}N_3O_3$: C 41.14, H 7.43; found: C 40.96, H 7.37.

2-{2-[2-(2-Azidoethoxy)ethoxy]ethoxy}ethanol (**11**$_4$): Yield 85%. ^{1}H-NMR ($CDCl_3$, 200 MHz): 3.73–3.56 (*m*, 14 H); 3.37 (*t*, J = 5.0, 2 H). Anal. calc. for $C_8H_{17}N_3O_4$: C 43.83, H 7.76; found: C 43.96, H 7.87.

2-(2-Aminoethoxy)ethanol (**12**$_2$). At r.t., **11**$_2$ (4.8 g, 36.6 mmol, 1 equiv.), PPh_3 (10.6 g, 1,1 equiv.), and H_2O (1 ml, 1.5 equiv.) in THF (40 ml) were stirred for 4 h. The soln. was directly evaporated and the residue chromatographed ($CHCl_3$/MeOH/Et_3N 3:3:1): **12**$_2$ as slightly yellow oil (3.8 g, 99%). TLC ($CHCl_3$/MeOH/Et_3N 3:3:1): R_f 0.3. ^{1}H-NMR ($CDCl_3$, 200 MHz): 3.66 (*t*, J = 4.0, 2 H); 3.53 (*t*, J = 4.0, 2 H); 3.48 (*t*, J = 4.8, 2 H); 2.83 (*t*, J = 4.8, 2 H). Anal. calc. for $C_4H_{11}NO_2$: C 45.71, H 10.48; found: C 45.51, H 10.35.

The same procedure was applied to **11**$_3$ and **11**$_4$ to give quantitatively **12**$_3$ and **12**$_4$.

2-[2-(2-Aminoethoxy)ethoxy]ethanol (**12**$_3$). ^{1}H-NMR ($CDCl_3$, 200 MHz): 3.69–3.47 (*m*, 10 H); 2.82 (*t*, J = 5.1, 2 H). Anal. calc. for $C_6H_{15}NO_3$: C 48.32, H 10.07; found: C 47.97, H 9.87.

2-{2-[2-(2-Aminoethoxy)ethoxy]ethoxy}ethanol (**12**$_4$). ^{1}H-NMR ($CDCl_3$, 200 MHz): 3.72–3.44 (*m*, 14 H); 2.79 (*t*, J = 4.9, 2 H). Anal. calc. for $C_8H_{19}NO_4$: C 49.74, H 9.84; found: C 49.37, H 9.57.

2-{2-[(tert-Butoxycarbonyl)amino]ethoxy}ethanol (**13**$_2$). KOH (0.88 g, 15.7 mmol, 1.1 equiv.) was added to **12**$_2$ (1.5 g, 1 equiv.) in H_2O (10 ml). The soln. was stirred at 0° and di(*tert*-butyl)dicarbonate (3.43 g, 1.1 equiv.) in dioxane (5 ml) was added dropwise. After 1 h, the soln. was allowed to warm to r.t. and stirred 4 h more. The mixture was extracted with CH_2Cl_2, the org. phase dried and evaporated, and the residue chromatographed (AcOEt/hexane 8:2): **13**$_2$ as colourless liquid (2.77 g, 95%). TLC (AcOEt): R_f 0.4. ^{1}H-NMR ($CDCl_3$, 200 MHz): 3.72 (*t*, J = 4.4, 2 H); 3.55 (*t*, J = 4.8, 2 H); 3.53 (*t*, J = 5.1, 2 H); 3.32 (*t*, J = 5.1, 1 H); 3.29 (*t*, J = 5.1, 1 H); 1.42 (*s*, 9 H). Anal. calc. for $C_9H_{19}NO_4$: C 52.68, H 9.27; found: C 52.61, H 9.25.

Compounds **13**$_3$ and **13**$_4$ were obtained in the same way.

2-{2-[2-[(tert-Butoxycarbonyl)amino]ethoxy}ethoxy}ethanol (**13**$_3$). Yield 93%. ^{1}H-NMR ($CDCl_3$, 200 MHz): 3.81–3.67 (*m*, 2 H); 3.64–3.58 (*m*, 6 H); 3.56 (*t*, J = 5.3, 2 H); 3.32 (*td*, J = 5.2, 5.2, 2 H); 1.44 (*s*, 9 H). Anal. calc. for $C_{11}H_{23}NO_5$: C 53.01, H 9.24; found: C 53.12, H 9.06.

2-{2-{2-[2-[(tert-Butoxycarbonyl)amino]ethoxy}ethoxy}ethoxy}ethanol (**13**$_4$). Yield 90%. ^{1}H-NMR ($CDCl_3$, 200 MHz): 3.74–3.57 (*m*, 12 H); 3.52 (*t*, J = 4.9, 2 H); 3.30 (*td*, = 5.0, 5.0, 2 H); 1.43 (*s*, 9 H). Anal. calc. for $C_{13}H_{27}NO_6$: C 53.24, H 9.21; found: C 53.00, H 9.07.

2-Aminoethyl (R)-2,3-Bis(oleoyloxy)propyl Hydrogen Phosphate (**14**$_1$). Et_3N (0.27 ml, 1.93 mmol, 1.2 equiv.) was added to $POCl_3$ (0.18 ml, 1.2 equiv.) in anh. THF (5 ml) at 0°. The mixture was treated dropwise with **8** (1 g, 1.6

mmol, 1 equiv.) dissolved in anh. THF (5 ml). After 1 h at 0°, the soln. was allowed to warm to r.t. and stirred for 3 h. Then it was cooled at 0°, and a soln. containing 2-aminoethanol (136 µl, 1.4 equiv.), Et_3N (0.54 ml, 2.4 equiv.), and THF (5 ml) was added dropwise. The mixture was stirred 4 h at 0° and 8 h more at r.t. before being filtered. H_2O (0.5 ml) was added to the filtrate that was stirred 3 h at r.t., evaporated, and purified by CC (CH_2Cl_2/EtOH 85:15 to 60:40): **14**$_1$ [31] as white crystalline powder (0.72 g, 64%). TLC (CH_2Cl_2/MeOH 8:2): R_f 0.4. ^{1}H-NMR ($CDCl_3$, 200 MHz): 5.36–5.16 (*m*, 5 H); 4.35 (*dd*, *J* = 12.2, 3.4, 1 H); 4.31–4.19 (*m*, 2 H); 4.14 (*dd*, *J* = 12.2, 6.4, 1 H); 4.06 (*td*, *J* = 7.8, 1.7, 2 H); 3.43–3.29 (*m*, 2 H); 2.33 (*td*, *J* = 7.1, 2.4, 4 H), 2.04–1.97 (*m*, 8 H), 1.62–1.55 (*m*, 4 H); 1.38–1.19 (*m*, 40 H); 0.88 (*t*, *J* = 6.4, 6 H).

*2-(2-Aminoethoxy)ethyl (*R*)-2,3-Bis(oleoyloxy)propyl Hydrogen Phosphate* (**14**$_2$). Et_3N (0.27 ml, 1.93 mmol, 1.2 equiv.) was added to $POCl_3$ (0.18 ml, 1.2 cquiv.) in anh. THF (5 ml) at 0°. The mixture was treated dropwise with **8** (1 g, 1.6 mmol, 1 equiv.) dissolved in anh. THF (5 ml). After 1 h at 0°, the soln. was allowed to warm to r.t. and stirred for 3 h. Then, it was cooled to 0°, and THF (5 ml) containing **13**$_2$ (0.46 g, 1.4 equiv.) and Et_3N (0.54 ml, 2.4 equiv.) were added dropwise. The mixture was stirred 4 h at 0°, then 8 h at r.t. H_2O (2 ml) was added and the soln. stirred overnight. The mixture was extracted with CH_2Cl_2, the extract dried and evaporated, and the residue purified by CC (CH_2Cl_2/MeOH 9:1): Boc-amino-protected phosphate as slightly yellow oil (1.0 g, 71%). TLC (CH_2Cl_2/MeOH 8:2): R_f 0.5. ^{1}H-NMR ($CDCl_3$, 200 MHz): 6.72 (*m*, 1 H); 5.36–5.30 (*m*, 4 H); 5.28–5.17 (*m*, 1 H); 4.38 (*dd*, *J* = 12.2, 3.7, 1 H); 4.14 (*dd*, *J* = 12.2, 7.2, 1 H); 4.04–3.87 (*m*, 4 H); 3.65–3.49 (*m*, 4 H); 3.33–3.13 (*m*, 2 H); 2.26 (*td*, *J* = 7.1, 3.5, 4 H); 2.11–1.96 (*m*, 8 H); 1.67–1.52 (*m*, 4 H); 1.43 (*s*, 9 H); 1.41–1.22 (*m*, 40 H); 0.88 (*t*, *J* = 6.2, 6 H).

The Boc-amino-protected phosphate (0.85 g, 0.95 mmol, 1 equiv.) was treated with CF_3COOH in CH_2Cl_2 (5 ml, CF_3CO_2H/CH_2Cl_2 1:9) for 2 h at r.t. The soln. was evaporated and directly purified by CC (CH_2Cl_2/MeOH 9:1): **14**$_2$ as white crystalline powder (0.59 g, 78%). TLC (CH_2Cl_2/MeOH 8:2): R_f 0.5. ^{1}H-NMR (CD_3OD, 200 MHz): 5.38–5.33 (*m*, 4 H); 5.30–5.24 (*m*, 1 H); 4.45 (*dd*, *J* = 11.1, 4.0, 1 H); 4.18 (*dd*, *J* = 8.0, 4.1, 1 H); 4.05–3.99 (*m*, 4 H); 3.77–3.71 (*m*, 4 H); 3.15 (*t*, *J* = 5.1, 2 H); 2.34 (*td*, *J* = 7.3, 3.4, 4 H); 2.13–1.95 (*m*, 8 H); 1.72–1.55 (*m*, 4 H); 1.46–1.22 (*m*, 40 H); 0.90 (*t*, *J* = 6.4, 6 H). FAB-MS (mNBA-TSA): 788.8 ([*M* – H]$^-$, expected 787.1)[2]).

Compounds **14**$_3$ and **14**$_4$ were obtained in the same way.

*2-[2-(2-Aminoethoxy)ethoxy]ethyl (*R*)-2,3-Bis(oleoyloxy)propyl Hydrogen Phosphate* (**14**$_3$). ^{1}H-NMR (CD_3OD, 200 MHz): 5.39–5.34 (*m*, 4 H); 5.30–5.24 (*m*, 1 H); 4.46 (*dd*, *J* = 11.2, 4.0, 1 H); 4.19 (*dd*, *J* = 8.0, 4.0, 1 H); 4.05–4.00 (*m*, 4 H); 3.77–3.70 (*m*, 8 H); 3.15 (*t*, *J* = 5.0, 2 H); 2.34 (*td*, *J* = 7.2, 3.4, 4 H); 2.14–1.97 (*m*, 8 H); 1.72–1.55 (*m*, 4 H); 1.48–1.25 (*m*, 40 H); 0.92 (*t*, *J* = 6.4, 6 H). FAB-MS (mNBA-TSA): 832.8 ([*M* – H]$^-$, expected 831.1)[2]).

*2-{2-[2-(2-Aminoethoxy)ethoxy]ethoxy}ethyl (*R*)-2,3-Bis(oleoyloxy)propyl Hydrogen Phosphate* (**14**$_4$). ^{1}H-NMR (CD_3OD, 200 MHz): 5.39–5.34 (*m*, 4 H); 5.31–5.24 (*m*, 1 H); 4.46 (*dd*, *J* = 11.2, 4.0, 1 H); 4.19 (*dd*, *J* = 8.1, 4.0, 1 H); 4.07–4.01 (*m*, 4 H); 3.86–3.68 (*m*, 12 H); 3.15 (*t*, *J* = 4.9. 2 H); 2.34 (*td*, *J* = 7.2, 3.4, 4 H); 2.14–1.96 (*m*, 8 H); 1.72–1.55 (*m*, 4 H); 1.48–1.25 (*m*, 40 H); 0.92 (*t*, *J* = 6.3, 6 H). FAB-MS (mNBA-TSA): 876.9 ([*M* – H]$^-$, expected 875.2)[2]).

*(*R*)-2,3-Bis(oleoyloxy)propyl 2-{2-[*N*-(11β,17α-Dihydroxy-3-oxoandrost-4-ene-17β-carbonyl)amino]ethoxy}ethyl Hydrogen Phosphate* (**1a**$_2$). A soln. of **14**$_2$ (53 mg, 67.2 µmol, 1 equiv.), *N*,*N*′-dicyclohexylcarbodiimide (20 mg, 1.4 equiv.), 4-(dimethylamino)pyridine (2 mg, 0.25 equiv.) and 11β,17α-dihydroxy-3-oxoandrost-4-ene-17β-carboxylic acid [22] [23] (23 mg, 1 equiv.) in CH_2Cl_2 (2 ml) was stirred for 6 h at r.t. The solvent was evaporated and the residue purified by CC (CH_2Cl_2/MeOH 9:1 to 7:3): **1a**$_2$ as glassy solid (59 mg, 78%). TLC (CH_2Cl_2/MeOH 8:2): R_f 0.5. ^{1}H-NMR ($CDCl_3/CD_3OD$ 1:1, 200 MHz): 5.75 (*s*, 1 H); 5.37–5.32 (*m*, 4 H); 5.29–5.24 (*m*, 1 H); 4.42 (*dd*, *J* = 12.1, 3.0, 1 H); 4.17 (*dd*, *J* = 12.0, 7.1, 1 H); 4.05–3.97 (*m*, 4 H); 3.75–3.56 (*m*, 4 H); 3.54–3.46 (*m*, 2 H); 2.60–1.00 (*m*, 18 H); 2.34 (*td*, *J* = 7.0, 2.5, 4 H); 2.12–1.93 (*m*, 8 H); 1.70–1.55 (*m*, 4 H); 1.42–1.20 (*m*, 40 H); 1.24 (*s*, 3 H); 0.90 (*t*, *J* = 6.4, 6 H); 0.76 (*s*, 3 H). FAB-MS (mNBA-TSA): 1116.9 ([*M* – H]$^-$, expected 1117.5).

Compounds **1a**$_1$, **1a**$_3$, **1a**$_4$, **1b**$_1$, **1b**$_2$, **1b**$_3$, and **1b**$_4$ were obtained following the same procedure (yields 76–84%).

Benzyl 2-Hydroxyacetate. Hydroxyacetic acid (5 g, 65.7 mmol, 1 equiv.) and Na_2CO_3 (5.52 g, 1 equiv.) were dried by azeotropic distillation with benzene in a *Dean-Stark* apparatus for 5 h. Benzene was removed under reduced pressure, and the sodium salt was suspended in anh. MeCN (50 ml). Anh. HMPA (2.3 ml, 0.2 equiv.), a catalytic amount of NaI and benzyl bromide (11.2 g, 1 equiv.) were added before refluxing for 3 days. The mixture was filtered, evaporated and chromatographed (Et_2O/C_6H_{14} 1:1): colourless liquid (8.5 g, 78%). TLC (Et_2O/hex-

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[2]) The MS was obtained from the sample in CD_3OD used to record the NMR spectrum, what results in exchanging the 3 acidic protons of the molecule by a D-atom. The resulting expected mass is in faet [*M* – H + 2]$^-$, what is observed.

ane 1:1): R_f 0.2. ^{1}H-NMR ($CDCl_3$, 200 MHz): 7.37 (*s*, 5 H); 5.23 (*s*, 2 H); 4.21 (*s*, 2 H). Anal. calc. for $C_9H_{10}O_3$: C 65.06, H 6.02; found: C 64.85, H 6.19.

Benzyl 2-(Methanesulfonyl)acetate (**15**). Benzyl hydroxyacetate (4 g, 24 mmol, 1 equiv.) and Et_3N (6.7 ml, 2 equiv.) in anh. Et_2O (50 ml) were treated dropwise at 0° with methanesulfonyl chloride (2.43 ml, 1.3 equiv.) for 2 h, then overnight at r.t. The soln. was filtered and the precipitate triturated with Et_2O (20 ml, by portions). The org. solns. were combined and evaporated and the residue chromatographed (Et_2O/hexane 1:1): **15** as colourless liquid (5.1 g, 87%). TLC (Et_2O/hexane 1:1): R_f 0.3. ^{1}H-NMR ($CDCl_3$, 200 MHz): 7.38 (*s*, 5 H); 5.24 (*s*, 2 H); 4.82 (*s*, 2 H); 3.20 (*s*, 3 H). Anal. calc. for $C_{10}H_{12}O_5S$: C 49.18, H 4.92; found: C 48.96, H 5.06.

2-[(17β-Hydroxyestra-1,3,5(10)-trien-3-yl)oxy]acetic Acid (**17**). For 2 h, **16** (302 mg, 1.1 mmol, 1 equiv.) was refluxed in anh. MeOH (10 ml) with NaOMe (62.6 mg, 1 equiv.). The solvent was evaporated and replaced by anh. MeCN/DMF 3:1 (20 ml). After addition of **15** (584 mg, 2.2 equiv.), the soln. was refluxed for 6 days. MeCN was evaporated and H_2O (5 ml) added before extraction with AcOEt. The org. phase was dried and evaporated to be chromatographed (Et_2O/hexane 1:1): benzyl ester of **17** as glassy solid (253 mg, 56%). TLC (Et_2O): R_f 0.6. ^{1}H-NMR ($CDCl_3$, 200 MHz): 7.38–7.36 (*m*, 5 H); 7.20 (*d*, J = 8.5, 1 H); 6.70 (*dd*, J = 8.5, 2.8, 1 H); 6.61 (*d*, J = 2.8, 1 H); 5.24 (*s*, 2 H); 4.64 (*s*, 2 H); 3.74 (*t*, J = 8.2, 1 H); 2.86–2.78 (*m*, 2 H); 2.35–1.12 (*m*, 13 H); 0.81 (*s*, 3 H).

A soln. of benzyl ester of **17** (120 mg, 0.28 mmol, 1 equiv.) in 1,2-dimethoxyethane/H_2O 1:1 (4 ml) was treated with KOH (32 mg, 2 equiv.) at r.t. for 3 h. The soln. was acidified with 10% HCl soln. and extracted with AcOEt. The org. phase was dried and evaporated and the residue chromatographed (CH_2Cl_2/EtOH 9:1 to 7:3): **17** as white crystalline powder (83 mg, 88%). TLC (CH_2Cl_2/EtOH 7:3): R_f 0.3. ^{1}H-NMR ($CDCl_3$, 200 MHz): 7.24 (*d*, J = 8.6, 1 H); 6.73 (*dd*, J = 8.5, 2.6, 1 H); 6.66 (*d*, J = 2.6, 1 H); 4.73 (*s*, 2 H); 3.93 (*t*, J = 8.3, 1 H); 2.41–2.13 (*m*, 3 H); 2.02–1.20 (*m*, 10 H); 0.81 (*s*, 3 H). ^{13}C-NMR ($CDCl_3$, 50 MHz): 181.9, 154.9, 138.4, 134.2, 126.7, 117.1, 115.0, 111.4, 87.1, 64.9, 49.5, 43.6, 43.4, 38.3, 36.6, 29.6, 27.1, 25.9, 23.1, 11.8. Anal. calc. for $C_{20}H_{26}O_4$: C 72.73, H 7.88; found: C 72.35, H 7.67.

(2R)-2,3-Bis(oleoyloxy)propyl 2-{2-{2-{2-[(17β-Hydroxyestra-1,3,5(10)-trien-3-yl)oxy]acetylamino}-ethoxy}ethoxy}ethoxy}ethyl Hydrogen Phosphate (**2**$_4$). For 5 h, **14**$_4$ (57.5 mg, 65.6 μmol, 1 equiv.), *N,N'*-dicyclohexylcarbodiimide (22.5 mg, 1.6 equiv.), benzotriazol-1-ol (13 mg, 1.5 equiv.), and **17** (31.4 mg, 1 equiv.) were allowed to react in CH_2Cl_2/CH_3COCH_3 1:1 (2 ml) for 5 h. The soln. was evaporated and chromatographed (CH_2Cl_2/EtOH 8:2 to 6:4): **2**$_4$ as white solid (39.4 mg, 43%). TLC (CH_2Cl_2/EtOH 8:2): R_f 0.3. ^{1}H-NMR ($CDCl_3/CD_3OD$ 1:1, 200 MHz): 7.23 (*d*, J = 9.2, 1 H); 6.76 (*dd*, J = 9.3, 3.0, 1 H); 6.68 (*d*, J = 3.0, 1 H); 5.37–5.32 (*m*, 4 H); 5.32–5.19 (*m*, 1 H); 4.50 (*s*, 2 H); 4.47–4.37 (*m*, 1 H); 4.23–4.12 (*m*, 1 H); 4.06–3.96 (*m*, 5 H); 3.71–3.60 (*m*, 12 H); 3.59–3.42 (*m*, 2 H); 2.92–2.77 (*m*, 2 H); 2.40–1.10 (*m*, 13 H); 2.34–2.27 (*m*, 4 H); 2.10–1.95 (*m*, 8 H); 1.70–1.52 (*m*, 4 H); 1.32–1.24 (*m*, 40 H); 0.90 (*t*, J = 6.4, 6 H); 0.79 (*s*, 3 H). FAB-MS (mTEA): 1186.8 ([M – H]$^-$, expected 1187.6).

Compounds **2**$_1$, **2**$_2$, **2**$_3$, **3**$_1$, **3**$_2$, and **3**$_4$ were obtained in the same way (yields 39–43%).

^{1}H-NMR spectra and MS of **1a**$_2$, **2**$_4$ and **14**$_{2-4}$, and ^{1}H-NMR spectrum of **7** are available as supplementary material.

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