Hydrogen abstraction from the C15 position of the cholesterol skeleton

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Abstract graphic
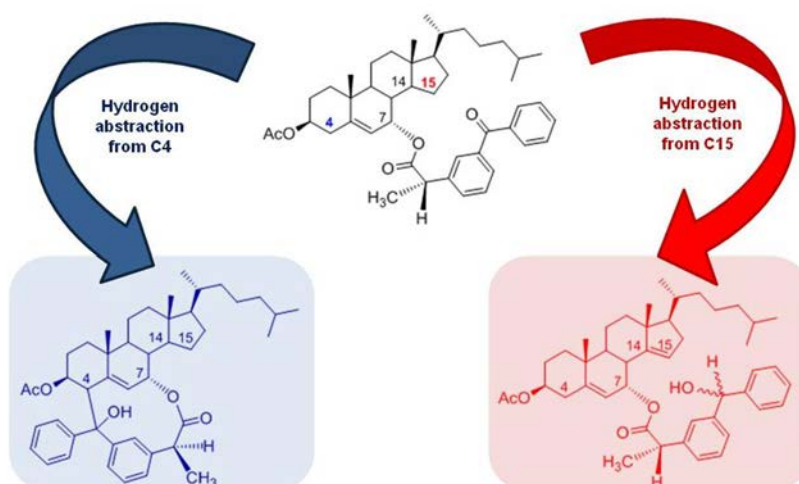
Abstract

Cholesterol (**Ch**) is an integral part of cell membranes, where it is prone to oxidation. In humans, oxidation of **Ch** is commonly linked to various pathologies like Alzheimer's, atherosclerosis and even cancer, which proceed *via* mechanisms involving enzymatic and free radical pathways. The latter begin with hydrogen abstraction (HA) from **Ch** by a reactive free radical. It has been established that the most efficient HA from **Ch** occurs at C7, although HA from C4 by peroxyl radicals has recently been observed. Conversely, HA from **Ch** positions other than the thermodynamically preferred C7 or C4 has never been reported. We have designed a **Ch** derivative where a benzophenone moiety is linked to C7 by a covalent bond. This mirrors a specific orientation of **Ch** within a confined environment. Product analysis and time-resolved spectroscopic studies reveal an unprecedented HA from C15, which is a thermodynamically unfavorable position. This indicates that a specific topology of reactants is crucial for the reactivity of **Ch**. The relative orientation of the reactants can also be relevant in biological membranes, where **Ch**, polyunsaturated fatty acids (PUFA) and numerous oxidizing species are confined in highly restricted and anisotropic environments.

Introduction

Cholesterol (**Ch**) is one of the most important building blocks of eukaryotic cells. This molecule carries out multiple functions in cell membranes: it controls membrane fluidity and permeability, composing rigid rafts to support various membrane proteins. Moreover, **Ch** plays a significant role in the biosynthesis of numerous hormones, vitamin D, and bile acids. Cholesterol is also one of the main targets for oxidation in cell

38 membranes. In humans, oxidation of **Ch** is commonly linked to various pathologies like

39 Alzheimer's, atherosclerosis and even cancer.[1–6] Such detrimental processes proceed *via*

40 distinct mechanisms, including enzymatic and free radical pathways. The latter begin

41 with hydrogen abstraction (HA) from **Ch** by a reactive free radical (Scheme 1) primarily

42 forming a **Ch**-derived radical.[7] The follow-up reaction with molecular oxygen leads to

43 peroxyl radicals that, in turn, can abstract hydrogen from another **Ch** molecule,

44 polyunsaturated fatty acids, or antioxidants.[8] Oxidation *via* radicals *in vivo* may be

45 caused by reactive oxygen species but also various photo-sensitive drugs like ketoprofen

46 that are capable of abstracting hydrogen atoms from biological targets like

47 polyunsaturated fatty acids (PUFA) and DNA inducing potentially damaging chemical

48 reactions. Indeed, ketoprofen[9] (2-(3-benzoylphenyl)propanoic acid), which is widely

49 used as non-steroidal anti-inflammatory drug (NSAID) with analgesic and antipyretic

50 effects, is able to absorb UVA light that can penetrate the middle layer of skin (the

51 dermis), inducing frequently phototoxic and photoallergic effects in patients.[10,11]

52 The primary HA from a steroid skeleton by photoexcited benzophenone has been largely

53 investigated by Breslow and coworkers many years ago.[12,13] They have synthesized

54 saturated dihydrosteroid derivatives (e.g. 3α-cholestanol) comprising a benzophenone

55 moiety. Based on product analysis of irradiated solutions, CD spectra, phosphorescence

56 lifetime, and molecular models, they could show that HA is directed mainly by the

57 topology of the steroid/benzophenone dyads. In contrast to dihydrosteroids, **Ch** carries

58 a double bond at C5-C6. In this latter case, it is well established that the most efficient

59 hydrogen abstraction occurs from C7 and C4.[14] Evidently, the bond dissociation energies

60 (BDE) of the allylic hydrogens, C7-H and C4-H are the lowest in the steroid core of **Ch**.

61 Therefore such a selectivity of hydrogen transfer can be explained by thermodynamic

control. However, in complex and inhomogeneous environments like membranes, even small molecules can be forced to a confined orientation.[15] This may cause entropic factors to become dominant. Farez *et al.* detected 15α-hydroxycholestene (15-HC) in patients with secondary progressive multiple sclerosis (SPMS).[16] This 15-oxysterol can be, in principle, formed as a result of an initial HA from C15 of cholesterol in cell membranes. Although the findings of Farez *et al.* are under debate,[17] it is important to find out whether a thermodynamically unfavorable HA from **Ch** can be overruled by topological confinement. In addition, taking into account that the steroidal skeleton, as well as C−H functionalization, have attracted the attention of synthetic chemists for decades, the present work is of considerable interest in the field of basic organic chemistry, since it is focused on selective activation of stronger C-H bonds in the presence of weaker ones.[18,19]
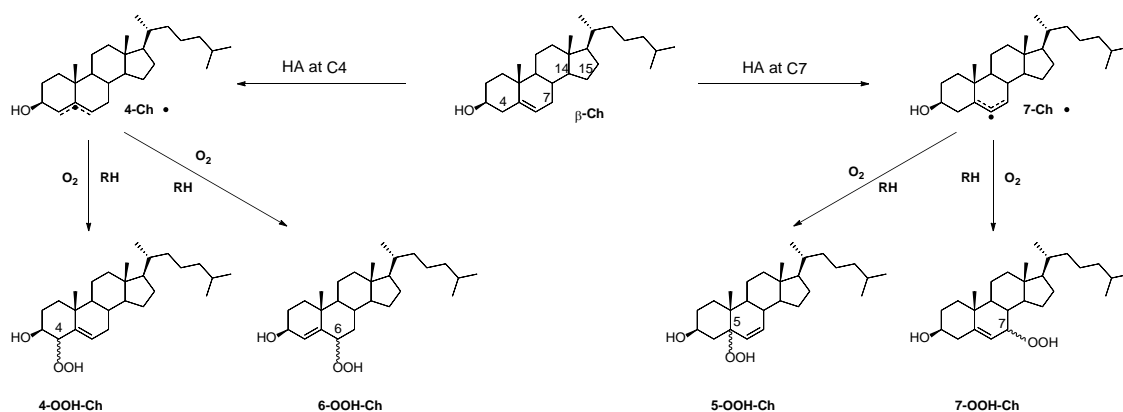
Scheme 1. Free radical oxidation of Cholesterol

We have shown that HA by the photo-excited triplet state of ketoprofen from cholesterol's core is predominantly controlled by entropic factors like the spatial

83   arrangement of reactants rather than by thermodynamic properties.[20] The question is

84   whether topology is a decisive factor for the selectivity of HA reactions in steroids, and

85   in this context we have designed and synthesized derivatives **1** and **2**. In these model

86   systems, the benzophenone fragment serves as a selective, photo-triggered

87   intramolecular hydrogen-abstracting agent with the reaction at C7 being prevented by

88   steric strain and conformational restrictions. It is our aim to shed light onto the role of

89   topology/entropy for the selectivity of the HA reaction. In a first step, steady-state UV

90   irradiation and product analysis provides access to the photo-induced conversions. To

91   avoid artifacts caused by undesired follow-up reactions of steady-state photolysis, we

92   have applied time-resolved methodology for substantiating the photo induced reaction

93   (Figure 1). To this end, we have utilized Laser Flash Photolysis (LFP) and Time-Resolved

94   Chemically Induced Dynamic Nuclear Polarization (TR-CIDNP).

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100   **1**   **2**

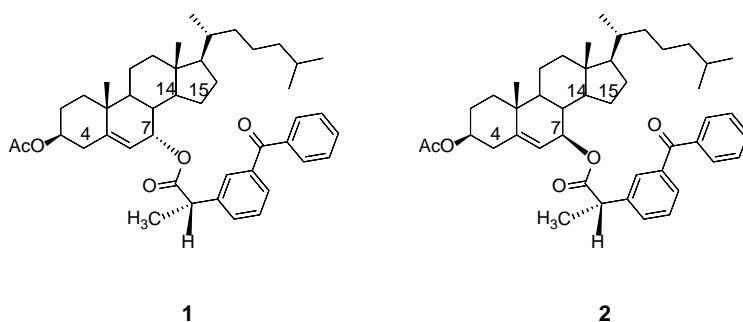101   **Figure 1** Chemical structures of diastereomeric dyads **1** and **2**

102

103   **Results and discussion**

104   We have arranged the presentation of our results in two separate sections, one

105   reporting on the photophysical/photochemical, the other on NMR/CIDNP-based

106   investigations supported with quantum mechanical calculations.

Steady state irradiation and flash photolysis

The diastereoisomeric esters **1** and **2** were prepared from acetylcholesterol (acetylCh) and (S)- ketoprofen ((S)-KP) following standard procedures[21] (see details in Supplementary Information). Steady state irradiations of **1** and **2** were carried out in dichloromethane solutions (*ca.* 3.5×10^{-3} M) under a nitrogen atmosphere, with a 400 W high-pressure mercury lamp as the light source. Even a long-term irradiation (8 hours) of dyad **2** did not lead to any products. In contrast, dyad **1** reacted readily yielding photoproducts **3** and **4** in similar quantities. Both **3** and **4** can only be formed *via* intramolecular HA by the excited triplet state of the benzophenone moiety from carbons C4 and C15 (or C14) of the steroid core. The overall photoreduction quantum yield for dyad **1** was determined using a related compound (S)-KP-3α-Ch as actinometer[22] and its value was found to be 0.43 (0.22 and 0.21 for the formation of photoproducts **3** and **4**, respectively). Scheme 2 shows a likely reaction mechanism. Structural differences between **1** and **2** that led to such a dissimilar reactivity will be discussed later.
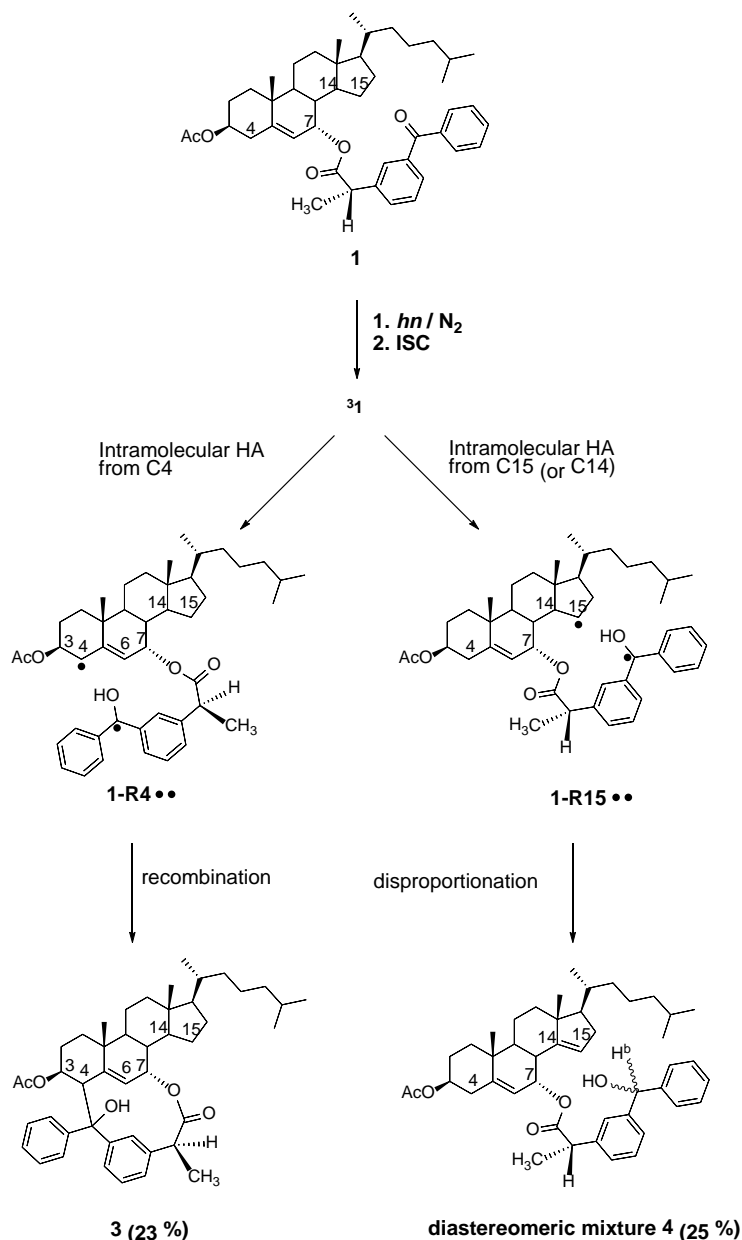
Scheme 2 Intramolecular HA and follow-up products formation in dyad **1**

Photo-excitation of **1** leads to its triplet excited state, which is confined to the benzophenone substituent and can abstract hydrogen atoms from the steroid fragment of the molecule. Hydrogen abstraction from the position C4 of **1** results in biradical **1-R4••**, subsequent recombination of the two radical centers in **1-R4••** yields photoproduct **3**. On the other hand, HA from C15 (or C14) produces **4** *via* intramolecular disproportionation of biradical **1-R15••** (or **1-R14••**). Remarkably, we did not observe

129 any disproportionation products after HA from C4, nor recombination products after HA

130 from C15 (C14).

131 We have established the structures of **3** and **4** unambiguously based on their NMR (^{1}H,

132 ^{13}C, HSQC and NOESY) and mass spectra. In particular, NOESY experiments of

133 photoproduct **3** provided the stereochemistry of the new stereogenic center at C4

134 generated upon photocyclization. The most relevant interaction was found between the

135 allylic proton at C4 and the hydrogen atoms of the phenyl group. Moreover, it should be

136 noted that no NOE effect was found between the hydrogens at C4 and C3; this indicates

137 that both protons are in a trans configuration (Figure 2).

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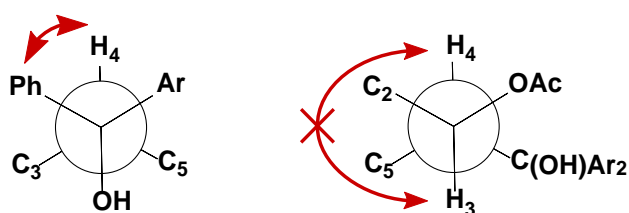
Figure 2. NOE interactions in **3**

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142 To gain insight into the photochemical processes at a shorter time scale, dyads **1** and **2**

143 were probed by LFP (λ_{exc} = 355 nm) in dichloromethane solutions, under an inert

144 atmosphere. The transient absorption spectrum of dyad **1**, obtained 0.3 μs after the

145 laser pulse (Figure 3), showed the exclusive presence of the benzophenone-based triplet

146 excited state with a characteristic maximum absorption at λ_{max} = 525 nm. It was not

147 possible to detect biradicals **1-R4••** and **1-R15••** (or **1-R14••**) on the microsecond time

148 scale because their steady state concentrations are negligible due to rapid follow-up

149 reactions. This is borne out by the observation that for dyad **2**, which does not display

150 intramolecular follow-up reactions, the ketyl radical (λ_{max} = 545 nm) was observed as a

151   result of a slow HA from the solvent, in addition to the triplet species. Kinetic analysis of

152   decay traces allowed determining the triplet lifetimes (τ_T) of **1** ($\tau_T = 0.60$ µs) and **2** ($\tau_T = $

153   1.67 µs). The latter matched the τ_T value of the reference **(S)-KP** in dichloromethane.[19]

154   The intramolecular quenching rate constant (i.e. the upper limit of the rate constant of

155   intramolecular HA) can be calculated using triplet lifetimes: $k_{iq} = 1/\tau_i - 1/\tau_0$, where τ_i and

156   τ_0 are triplet lifetimes of dyad and reference compound (**(S)-KP**, $\tau_0 = 1.6$ µs)

157   correspondingly. For dyad **1** the upper limit is 1.07×10^6 s^{-1} for either H atoms at C4 and

158   C15 (or C14). This strongly indicates that under UV irradiation **1** undergoes

159   intramolecular hydrogen abstraction that is in full agreement with the results of product

160   analysis after the longer irradiation times. The k_{iq} obtained for **1** was found to be one

161   order of magnitude lower than that determined for the intramolecular process in

162   compounds containing benzophenone and 1,4 cyclohexadienes as hydrogen donors.[23]
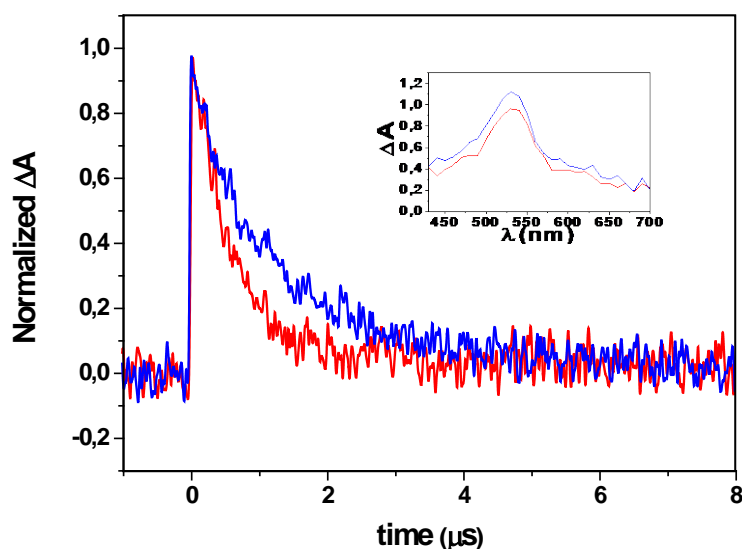
163

164   **Figure 3.** Normalized transient decays for **1** (red) and **2** (blue) monitored at 620 nm in CH$_2$Cl$_2$. Insert:

165   transient absorption of **1** and **2** (blue) recorded 0.3 µs after the laser flash.

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Time-resolved photo-CIDNP and quantum mechanical calculations

Photo-CIDNP is a powerful NMR-based method to follow reactions based on the initial formation of radical (ion) pairs. These radical pairs react *via* pathways that depend on the electron spin states of the reaction partners.[24,25] Since nuclear spin states are coupled to the electron spins, this spin-dependent reactivity leads to non-Boltzmann distributions of nuclear spin states. According to the long lifetimes of the non-Boltzmann populated nuclear states the NMR spectra of the products based on the primary radical pair indicate strongly enhanced (up to factor 10^6) emissive ('enhanced emission') or absorptive ('enhanced absorption') signals. The intensity of the NMR signals can be translated into the structure of the intermediate free radicals. Photo-CIDNP can be performed in a time-resolved fashion also revealing kinetic information on free radical reactions.[26]

Here we used TR photo-CIDNP in combination with quantum mechanical calculations to distinguish between reaction pathways and intermediate free radicals formed by **1** after photo-excitation in different solvents.

Photo-^{1}H CIDNP and ^{1}H NMR spectra of **1** in dichloromethane together with selected signal assignments are shown in Figure 4. The experimental design guarantees the suppression of background (equilibrium) NMR signals in CIDNP spectra, thus only pure CIDNP polarizations with no contamination are observed. The appearance of CIDNP effects *per se* indicates that upon photo-excitation **1** undergoes a free radical reaction that starts with a formation of a correlated radical pair or a biradical that is in concert with the results obtained by LFP. Major polarizations in the CIDNP spectrum of **1** belong to aliphatic protons in the steroid core (S, 0 – 2.5 ppm) and aromatic protons of the benzophenone moiety (BP, 7 – 8 ppm). This denotes that in the intermediate free

191   radicals both steroid and benzophenone parts carry significant unpaired electron spin

192   density. The NMR transitions of the individual protons in the S and BP groups (Figure 4)

193   severely overlap with each other and are difficult to be analyzed further; however, there

194   are two characteristic polarizations in the CIDNP spectrum of **1** that can bring forth

195   additional information on the structure of reacting free radicals or biradicals.

196   Polarizations at 5.24 ppm ('enhanced emission') and 5.82 ppm ('enhanced absorption')

197   are unambiguously assigned to protons H15 and Hb in photoproduct **4** (Scheme 2, Figure

198   4). Additionally, two small polarizations are present at 3.1 ppm ('enhanced absorption').

199   They presumably belong to the proton at C4 in recombination product **3**.
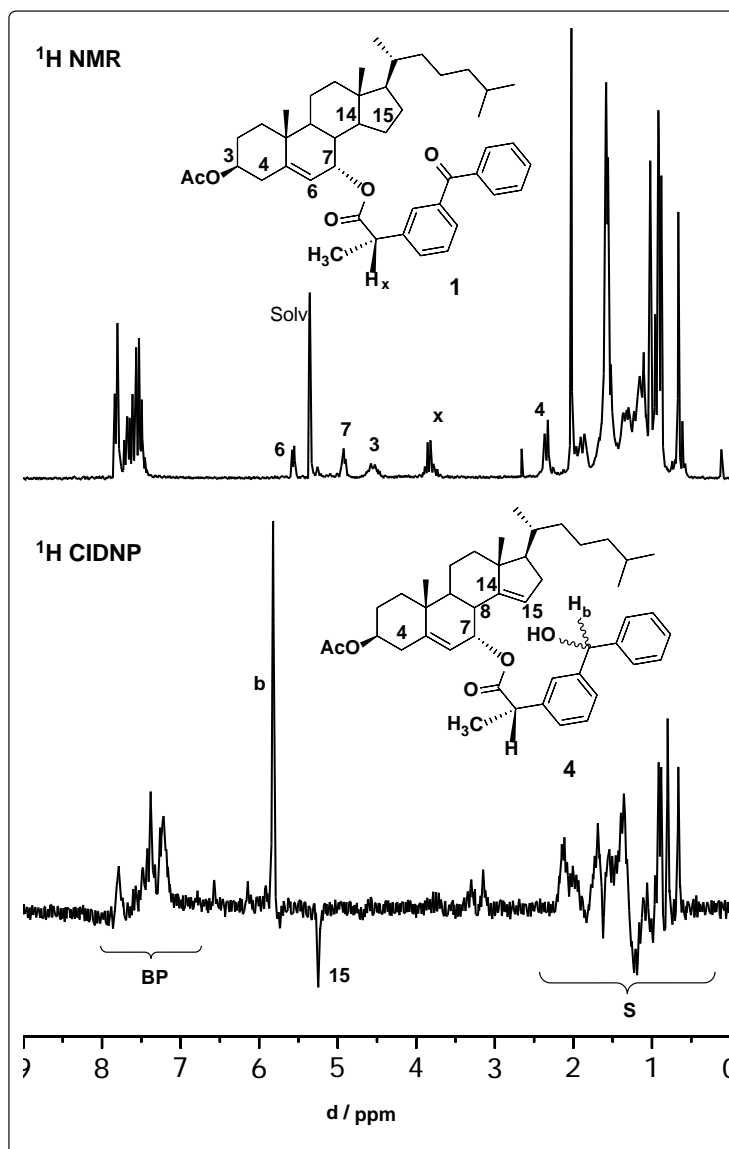
Figure 4. ^{1}H NMR (upper trace) and TR-CIDNP (bottom trace) spectra of **1** in dichloromethane-d$_2$. CIDNP spectrum is recorded 2 μs after the laser flash (8 ns, 355 nm)

The magnitude of the CIDNP polarizations strongly depends on a hyperfine coupling constant of corresponding nuclei in free radicals/biradicals where these polarizations were generated.[27,28] The very short time-delay between laser flash and observing radio-frequency pulses allows observation of almost exclusively CIDNP polarizations that are stored in a cage product formed immediately from the initial radical pair. Thus, the lines attributed to H15 and Hb in **4** (Figure 4) can be utilized to verify **1-R4••** and **1-R15••**.
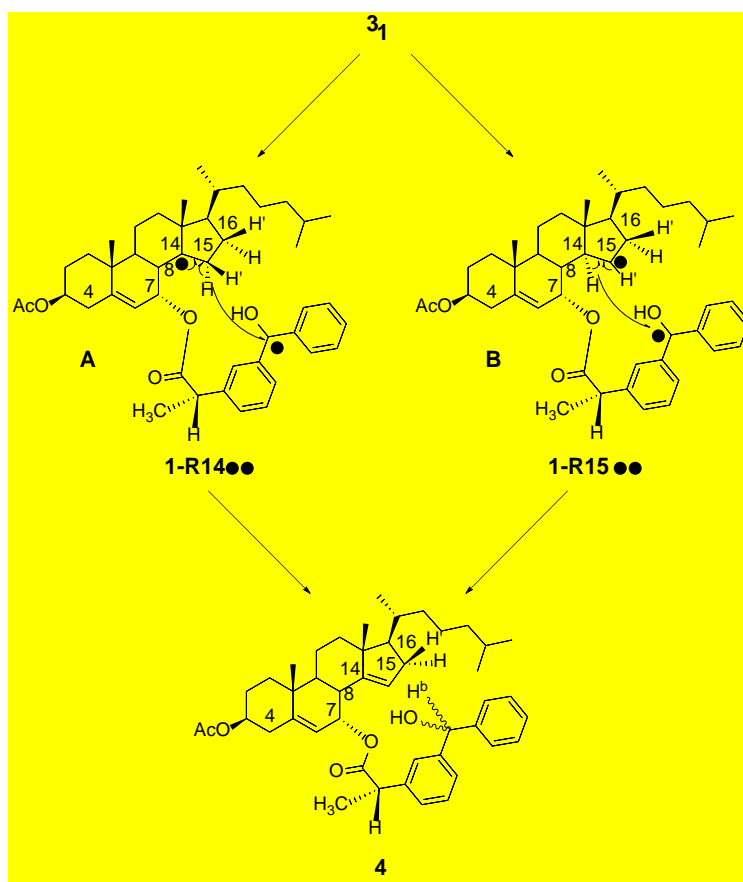
Scheme 3. Distinct reaction pathways to produce **4**

While **3** is the result of the intramolecular hydrogen transfer from C4 carbon of the

steroid moiety, **4** can be formed *via* HA from C15 or C14 (Scheme 3). These reaction

pathways cannot be discriminated by product analysis and LFP. However, intermediate

biradicals **1-R14••** and **1-R15••** should possess different unpaired spin distributions that

are reflected in different hyperfine couplings of protons in the molecules. This must lead

to different polarizations in CIDNP spectra. We have calculated hyperfine coupling

constants in both possible biradicals **1-R14••** and **1-R15••** that could be formed upon

HA from C14 and C15 correspondingly. The results for the selected protons are shown

in the radar graph on the Figure 5.

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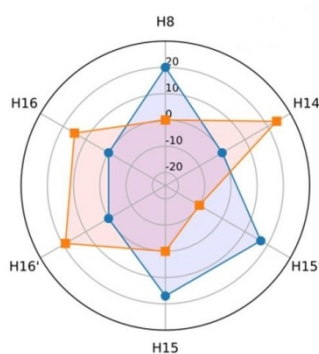
Figure 5 Calculated (B3LYP/TZVP) hfcs (in Gauss) of selected ^{1}H nuclei (for numbering, see Scheme 3) in biradicals **1-R14••** (dots, blue) and **1-R15••** (squares, orange)

Thus, it is clear that both biradicals have quite distinct hyperfine patterns. Hyperfine coupling constants that are plotted in Figure 5 are very different for matching protons in **1-R14••** and **1-R15••** (H8, H14, H15', H15, H16' and H16). In CIDNP spectrum upon irradiation of **1** polarizations of H8, H16 and H16' are overlapped with polarizations of different alkyl protons in parent **1** and/or in **3** and cannot be treated individually. In contrast, polarizations H15 and Hb (H15 and H15' in **1-R14••**; H14 and H15' in **1-R15••)** as we pointed out before, are clearly distinguishable (Figure 4).

There are two possible extreme cases in the photoreaction of **1** where **4** is exclusively formed *via* pathway **A** or **B** (Scheme 3). In the first case (path **A**) the ratio of the polarizations of Hb and H15 in **4** will be close to the ratio of the corresponding protons in **1-R14••** ($P_{Hb}/P_{H15} \approx hfc_{H15'}/hfc_{H15}$=+17 Gauss/+17 Gauss = +1). Moreover, since both protons have hyperfine coupling constants of the same sign, their signals will be polarized in the same direction. In the second case (path **B**), all the CIDNP effect is generated in biradical **1-R15••** and the ratio of the abovementioned polarizations will

238   be $P_{Hb}/P_{H15} \approx$ hfc $_{H14}$/hfc $_{H15'}$=+24 Gauss/−10 Gauss = −2.4 with opposite directions of

239   polarization. If **4** is simultaneously produced *via* both pathways the ratio of polarizations

240   will be determined by the superposition of these extreme scenarios. The P_{Hb}/P_{H15}

241   measured from the CIDNP spectrum (Figure 4) is 2.5, which is very close to the prediction

242   for the pathway B. This leads us to the conclusion that in dichloromethane **4** is produced

243   solely *via* hydrogen abstraction from C15 on the timescale of the CIDNP experiment. This

244   contrasts with the results obtained by Breslow and coworkers for other steroidal

245   systems (although lacking the important unsaturation between C5 and C6). He showed

246   the deuteration at position C15 of the steroid moiety, that the primary hydrogen

247   abstraction takes place exclusively from C14. The difference in Breslow's

248   benzophenone-coupled steroids is that linkers attached at the position 3α of the steroid

249   core were not long enough for the ketone chromophore to reach the hydrogen atom at

250   C15. In **1** the benzophenone moiety can get close to both C14 and C15; this explains HA

251   from C15 but leaves the question as to such a high selectivity towards hydrogen transfer

252   from C15 open. The photoproduct **3** that according to product analysis is formed

253   alongside **4** by the HA from C4 and subsequent intramolecular recombination of

254   biradical **1-R4••**, should also manifest itself in the CIDNP spectrum of **1**. The 'hyperfine

255   signature' of **1-R4••**, where the CIDNP effects were generated, shows that major

256   polarizations in **3** should stem from protons at carbons C3, C4, C6 and C7 of **1-R4••** since

257   they possess the most prominent hyperfine coupling constants according to our

258   quantum mechanical calculations (Figure 6). Unexpectedly, none of them apart from H4

259   (3.1 ppm) gives any significant polarization. This can be rationalized by the longer

260   (relative to **1-R14••** and **1-R15••**) lifetime of **1-R4••** and, possibly, shorter paramagnetic

261   relaxation times of protons H3, H4, H6 and H7 in the biradical. These factors can lead to

262   a vanishing of CIDNP polarizations even before the recombination of the biradical

263   occurs.
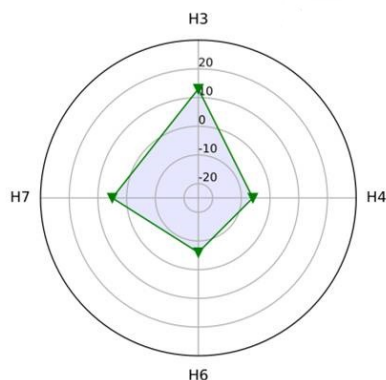
Figure 6. Calculated (B3LYP/TZVP) hfcs (in Gauss) of selected ^{1}H nuclei (for numbering, see Scheme 3) in biradical **1-R4••**(green)

268   Dyad **2** did not show any CIDNP effects. This is in full agreement with the product

269   analysis and can be understood in terms of the molecular structure. In **1**, the

270   benzophenone chromophore can adopt a conformation reaching H4, H14 and H15. This

271   is not feasible in **2**, where the benzophenone moiety resides above the steroid skeleton

272   deflecting the ketone oxygen from those protons.

273   According to DFT calculations, the bond dissociation energy (BDE) of the secondary

274   hydrogen at C15 is *ca.* 26 kJ/mol higher than that of C14-H (tertiary hydrogen). If only

275   enthalpic control would rule the reactivity, the hydrogen transfer should exclusively

276   happen from C14. The fact that we exclusively observe abstraction of C15-H reveals

277   entropic/topologic factors being decisive for the hydrogen abstraction.

278   Solvation has a marked effect on the shape of flexible and polar molecules.[29] In this

279   respect, we performed (in addition to CD$_2$Cl$_2$) ^{1}H TR-CIDNP experiments in toluene-d$_8$,

280   CDCl$_3$, and CD$_3$CN. Table 1 presents the contributions (in %) of HA from C14 *vs*. C15 taken

281   from the CIDNP intensities determined from product **4**. Whereas in toluene the

282   hydrogen is almost exclusively abstracted from C15 (> 90%), the use of chloroform shifts

283   the ratio between C14-H/C15-H to 1:4 and acetonitrile switches it to 2:3. These

284   observations are in line with a solvent-induced alteration of the preferred conformation

285   of dyad **1**.

286   **Table 1.** Contributions of HA from C14 and C15 into the formation of **4** from **1** in different solvents

287   calculated from TR CIDNP spectra for details, see the Supporting Information).

Solvent	Polarity index[30]	HA from C14 (%)	HA from C15 (%)
Toluene-d$_8$	2.4	< 10	> 90
CD$_2$Cl$_2$	3.1	< 10	> 90
CDCl$_3$	4.1	20	80
CD$_3$CN	5.8	40	60

288

Conclusion

290   Many biological reactions of cholesterol derivatives are ascribed to reactions in which

291   hydrogen abstraction is involved. Our investigations on dyads **1** and **2** consisting of a **Ch**

292   core and benzophenone show that in addition to the thermodynamically favored H-

293   abstraction at C4, products and radicals based on HA from C15 take place. Such a

294   regioselectivity is not observed in bimolecular reactions of **Ch** and benzophenone in

295   solution. This indicates that a specific orientation of the reactants is crucial for a site-

296   selective reactivity of **Ch**. A confined arrangement of the reactants is very likely to exist

297   in biological membranes containing **Ch**. Our observations are in agreement with clinical

298   studies[16] pointing to the formation of 15α-hydroxycholestene. Accordingly, our

299   investigation reveals a specific topology being decisive for the reactivity in restricted

300   (anisotropic) biological environments. Finally, selective activation of the strong C15-H

301   bond in the presence of a weaker C7-H allylic bond is of great interest in the C-H

302   functionalization research.

Experimental section

General

305   AcetylCh and (*S*)-KP were commercially available. Other commercial reagents and

306   solvents were used directly without further purification. One- (^{1}H and ^{13}C) and two-

307   dimensional (HSQC and NOESY) NMR spectra were recorded in CDCl$_3$ as solvent on a

308   Bruker AC-300; NMR chemical shifts are reported in ppm downfield from an internal

309   solvent peak. All reactions were monitored by analytical TLC with silica gel 60 F254

310   revealed with ammonium molybdate reagent. The residues were purified through silica

311   gel 60 (0.063–0.2 mm). Exact mass was obtained by TripleTOF™ 5600 LC/MS/MS System,

312   (AB SCIEX), equipped with an electrospray source. Thus, 7α-OH-acetylCh or 7β-OH-

313   acetylCh were synthesized from acetylCh following procedures reported in previous

314   publications. Their ^{1}H-NMR and ^{13}C-NMR signals coincide with those already described

315   in the literature.[31,32]

Synthesis of dyads 1 and 2

317   To a solution of (S)-KP (215 mg, 0.84 mmol) in CH$_2$Cl$_2$ (5 mL) dicyclohexylcarbodiimide

318   (DCC, 320 mg, 1.54 mmol) was added in small portions, and the mixture was stirred at

319 0°C for 30 min. Then, a solution of 7α-OH-acetylCh or 7β-OH-acetylCh (340 mg, 0.77

320 mmol) in CH_2Cl_2 (7 mL) and 4-dimethylaminopyridine (DMAP, 10 mg, 0.08 mmol) was

321 added, and the mixture was kept under stirring overnight at the same temperature. The

322 reaction mixture was then filtered through a pad of Celite®. The resulting filtrate was

323 washed with brine and water, dried over Na_2SO_4 and evaporated. The residue obtained

324 was purified by column chromatography (eluent: dichloromethane-hexane-

325 dichloromethane acetate 90:5:5 v/v/v) to give dyad **1** (355 mg, 0.52 mmol, 68%) and

326 dyad **2** (405 mg, 0.59 mmol, 77 %).

327 **(3S,7S,10R,13R,17R)-3-acetoxy-10,13-dimethyl-17-((R)-6-methylheptan-2-yl)2,3,4,7,8,**

328 **9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[*a*]phenanthren-7-yl (2S)-2-**

329 **(3-benzoylphenyl)propanoate (1).** ^{1}H-NMR (300 MHz, $CDCl_3$) δ = 7.35-7.74 (m, 9H, Ar*H*),

330 5.48 (m, 1H, C6-*H*), 4.84 (m, 1H; C7-*H*), 4.48 (m, 1 H; C3-*H*), 3.74 (q, *J* = 7.2 Hz, 1H; CH_3-

331 C*H*-CO), 2.25 (m, 2H; C4-*H_2*), 1.96 (s, 3H; *CH_3*), 0.98-1.94 (complex signal, 24H), 1.49 (d,

332 *J* = 7.2 Hz, 3H; *CH_3*), 0.91 (s, 3H; *CH_3*), 0.82 (d, *J* = 6.6 Hz, 3H; *CH_3*), 0.80 (d, *J* = 6.6 Hz, 3H;

333 *CH_3*), 0.79 (d, *J* = 6.6 Hz, 3H; *CH_3*), 0.54 (s, 3H; *CH_3*); [13]C{[1]H} (75 MHz, $CDCl_3$) δ = 196.3,

334 173.4, 170.3, 147.1, 140.8, 137.6, 132.3, 131.7, 130.0, 129.4, 129.1, 128.3, 120.3, 73.2,

335 68.9, 55.9, 49.1, 45.9, 43.2, 42.1, 39.5, 39.1, 37.8, 37.3, 36.6, 36.1, 35.9, 35.8, 28.0, 27.4,

336 24.0, 22.8, 22.5, 21.3, 20.7, 18.7, 18.1, 17.9, 11.4. HRMS (EI): *m/z* calcd for $C_{45}H_{61}O_5$

337 $(M+H)^+$: 681.4514; found: 681.4492.

338 **(3S,7R,10R,13R,17R)-3-acetoxy-10,13-dimethyl-17-((R)-6-methylheptan-2yl)2,3,4,7,8,**

339 **9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[*a*]phenanthren-7-yl (2S)-2-**

340 **(3-benzoylphenyl)propanoate (2)**. ^{1}H-NMR (300 MHz, $CDCl_3$) δ = 7.18-7.82 (m, 9H; Ar*H*);

341 5.24 (m, 1H; C6-*H*), 4.98 (m, 1H; C7-*H*), 4.61 (m, 1H; C3-*H*), 3.74 (q, *J* = 7.2 Hz, 1H; CH_3-

342   C*H*-CO), 0.67-2.37 (complex signal, 29H), 2.05 (s, 3H; C*H₃*), 1.55 (d, *J* = 7.2 Hz, 3H; C*H₃*),

343   1.09 (s, 3H; C*H₃*), 0.88 (d, *J* = 6.6 Hz, 3H; C*H₃*), 0.87 (d, *J* = 6.3 Hz, 3H; C*H₃*), 0.59 (s, 3H;

344   C*H₃*); $^{13}C\{^1H\}$ (75 MHz, CDCl₃) δ = 196.4, 173.8, 170.3, 144.5, 140.6, 137.7, 137.6, 132.4,

345   131.8, 130.0, 129.7, 129.0, 128.4, 128.3, 122.0, 73.2, 55.4, 53.4, 48.2, 45.7, 42.7, 39.5,

346   39.3, 38.2, 37.6, 36.5, 36.4, 36.3, 36.1, 35.7, 31.2, 30.9, 29.7, 28.0, 27.6, 25.1, 23.8, 22.8,

347   22.5, 21.3, 21.1, 18.9, 18.6, 18.2, 11.7. HRMS (EI): *m/z* calcd for $C_{45}H_{61}O_5$ (M+H)⁺:

348   681.4514; found: 681.4495.

349   **Steady-state photolysis of dyads 1 and 2**

350   Deaerated dichloromethane (40 mL) solutions of **1** or **2** (100 mg, 0.15 mmol) were

351   irradiated for 8 h through Pyrex with a 400 W medium pressure mercury lamp. After

352   that, the reaction mixtures were concentrated under reduced pressure, and the

353   photomixtures were submitted to silica gel column chromatography, using

354   hexane/dichloromethane/ethyl acetate (85:10:5 v/v/v) as eluent, affording the

355   photoproducts **3** (23 %) and **4** (diastereomeric mixture, 25 %); the rest was mixture of

356   several minor unidentified products, together with polymeric material. Moreover, to

357   determine the photoreduction quantum yield of dyad **1** in dichloromethane, (S)-KP-

358   3α–Ch was used as actinometer, with a quantum yield of 0.47.[22] For this purpose,

359   solutions of **1** and the actinometer were photolyzed under deaerated conditions using

360   a multilamp photoreactor model LZC-4 (Luzchem, Canada) equipped with 8 lamps ($λ_{max}$

361   = 350 nm, Gaussian distribution) and monitored by UV-spectrophotometry following the

362   decrease in the absorption at 254 nm. Thus, the quantum yield was calculated from the

363   slope of the plot absorbance at 254 nm *versus* irradiation time.

. ^{1}H-NMR (300 MHz, CDCl$_3$) δ = 7.71 (m, 1H; Ar*H*), 7.22-7-39 (m, 6H; Ar*H*), 7.12 (m, 1H; Ar*H*), 6.51 (s, 1H; Ar*H*), 5.47 (d, *J* = 5.1 Hz, 1H; C6-*H*), 4.53 (m, 1H; C7-*H*), 4.47 (m, 1H; C3-*H*), 3.70 (q, *J* = 7.5 Hz, 1H; CH$_3$-C*H*-CO), 3.05 (m, 1H; C4-H), 2.22 (m, 2H; C2-*H$_2$*),1.95 (s, 3H; C*H$_3$*), 0.42-1.90 (complex signal, 22H), 1.46 (d, *J* = 7.5 Hz, 3H; C*H$_3$*), 1.07 (d, *J* = 7.2 Hz, 3H; C*H$_3$*), 0.84 (s, 3H; C*H$_3$*), 0.81 (d, *J* = 6.6 Hz, 6H; 2 × C*H$_3$*), 0.60 (s, 3H; C*H$_3$*), -0.45 (m, 1H; C*H*); <mark>[13]C{[1]H}</mark> (75 MHz, CDCl$_3$) δ = 175.6, 170.4, 148.1, 146.9, 143.2, 138.7, 130.0, 129.8, 128.4, 128.3, 128.1, 127.8, 126.8, 126.4, 122.7, 120.4, 82.1, 73.2, 69.1, 55.9, 45.7, 44.0, 43.9, 43.7, 42.9, 39.4, 38.4, 37.6, 37.2, 36.4, 36.3, 35.3, 32.5, 30.8, 28.1, 27.4, 26.2, 22.8, 22.7, 21.3, 20.6, 20.0, 17.9, 14.3, 14.2. HRMS (EI): *m/z* calcd for C$_{45}$H$_{61}$O$_5$ (M+H)$^+$: 681.4514; found: 681.4518.

^{1}H-NMR (300 MHz, CDCl$_3$) δ = 7.08 – 7.32 (m, 9H; Ar*H*), 5.73 (m, 1H; Ph-C*H*-OH), 5.47 (m, 1H; C6-*H*), 5.12 (m, 1H; C15-*H*), 4.90 (m, 1H; C7-*H*), 4.36 (m, 1H; C3-*H*), 3.64 (q, *J* = 7.2 Hz, 1H; CH$_3$-C*H*-CO), 2.94 (m, 1H; C4-*H*), 2.82 (m, 1H; C4-*H*), 0.56 – 2.28 (complex signal, 35H), 1.95 (d, *J* = 1.8 Hz, 3H; C*H$_3$*), 0.79 (d, *J* = 6.6 Hz, 3H; C*H$_3$*), 0.78 (d, *J* = 6.6 Hz, 3H; C*H$_3$*), 0.67 (s, 3H; C*H$_3$*); <mark>[13]C{[1]H}</mark> (75 MHz, CDCl$_3$) δ = 174.0, 173.9, 170.8, 170.7, 160.5, 160.4, 146.9, 146.8, 144.4, 144.3, 144.1, 140.7, 139.3, 128.6, 128.5, 128.4, 128.3, 127.4, 127.3, 127.0, 126.6, 126.5, 125.6, 125.5, 125.2, 125.1, 120.6, 120.5, 120.4, 120.3, 114.1, 76.0,

387   75.9, 73.5, 73.4, 68.9, 68.8, 50.3, 50.2, 46.9, 45.6, 45.5, 43.5, 43.4, 39.2, 37.8, 37.5, 36.8,

388   36.6, 36.5, 34.7, 34.5, 32.2, 31.9, 30.9, 29.7, 27.9, 27.5, 27.4, 25.3, 22.7, 22.6, 21.9, 21.8,

389   21.4, 20.5, 18.1, 17.7, 17.5, 15.8, 14.1. HRMS (EI): m/z calcd for $C_{45}H_{61}O_5$ (M+H)$^+$:

390   681.4514; found: 681.4507.

391   **Laser flash photolysis measurements**

392   A pulsed Nd:YAG laser was used for excitation at 355 nm. The single pulse was ~10 ns

393   duration and the energy raning from 10 to 1 mJ pulse^{-1}. The LFP system consisted of the

394   pulsed laser, the Xe lamp, a monochromator and a photomultiplier made up of a tube,

395   housing and power supply. The output signal from the oscilloscope was transferred to a

396   personal computer. All experiments were performed at room temperature under

397   anaerobic conditions. The samples were dissolved in dichloromethane to have an

398   absorbance *ca*. 0.30 at 355 nm.

399   **CIDNP and quantum mechanical calculations of hyperfine coupling constants**

400   ^{1}H CIDNP spectra were recorded on a 200 MHz Bruker AVANCE DPX spectrometer.

401   Irradiation was carried out by using a frequency-tripled Quantel Nd:YAG Brilliant B laser

402   (10 Hz, 355 nm, *ca*. 90 mJ per pulse, pulse width *ca*. 8 ns). The following pulse sequence

403   was used: presaturation (waltz16) – laser flash – RF detection pulse (2 μs) – free

404   induction decay. Dark spectra (background) - the same sequence without the laser flash

405   - were always recorded to assure the effective suppression of background NMR signals.

406   The concentrations of **1** and **2** were 0.01 M. To exclude oxygen, samples were bubbled

407   with Argon for 5 minutes prior to experiments. Hyperfine coupling constants of free

408   radicals were calculated using the Gaussian 09[33] package. All calculations (geometry

409 optimizations and single-point calculations) were conducted at the B3LYP[34,35] level of

410 theory with the TZVP[36] basis set.

411 **Calculation of the contributions of 1-R14•• and 1-R15•• biradicals into CIDNP**

412 **polarizations of H15 and Hb protons.**

413 Polarizations of Hb (P_{Hb}) and H15 (P_{H15}) can be measured directly from the spectrum and

414 their ration is:

$$415 \qquad \frac{P_{Hb}}{P_{H10}} = -2.4 \qquad (1)$$

416 Polarization of Hb is constructed from contributions of **1-R14••** and **1-R15••** which are

417 proportional to hyperfine coupling constants of corresponding protons in those

418 biradicals and:

$$419 \qquad P_{Hb} = C \times a_{H14}^{R15} + (1 - C) \times a_{H15}^{R14} \qquad (2)$$

420 Where a_{H14}^{R15} and a_{H15}^{R14} are hiperfine coupling protons H14 and H15 in biradicals **1-R15••**

421 and **1-R14••** correspondingly. Analogously the polarization of H15 is represented:

$$422 \qquad P_{H15} = C \times a_{H15}^{R15} + (1 - C) \times a_{H15}^{R14}, \qquad (3)$$

423 The system of equations 1-3 can be solved with respect to proportionality constant C

424 which gives us contributions of 2 different biradicals into CIDNP polarizations.

425 <mark>**Associated content**</mark>

426 <mark>**Supporting Information**</mark>

427 <mark>The Supporting Information is available free of charge on the…</mark>

428 <mark>Scheme of the synthesis of dyads 1 and 2 (Scheme S1); ^{1}H and ^{13}C-NMR spectra of **7-**</mark>

429 <mark>**oxo-acetylCh**; ^{1}H and ^{13}C-NMR spectra of **7α-OH-acetylCh**; ^{1}H and ^{13}C-NMR spectra of</mark>

430 **7β-OH-acetylCh;** ^{1}H, ^{13}C-NMR and DEPT spectra of dyad **1**; ^{1}H, ^{13}C-NMR and DEPT spectra

431 of dyad **2**; ^{1}H, ^{13}C-NMR, DEPT, HSQC and NOESY spectra of photoproduct **3**; ^{1}H, ^{13}C-NMR,

432 DEPT, HSQC and NOESY spectra of photoproduct **4**; calculations B3LYP/TZVP optimized

433 model for dyad **1** and **2**.

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