Steroid-fullerene adducts from Diels–Alder reactions: characterization and the effect on the activity of Ca^{2+}-ATPase

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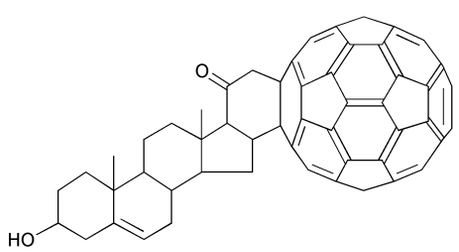
Two new fullerene steroids (**1** and **6**) have been prepared by the Diels–Alder reaction of silyloxydienes (**2** and **3**) with [60]fullerene, followed by hydrolysis of the silyl enol ether under acidic conditions. The isolation, characterization, UV-Vis and CD absorption studies of the adducts are presented. A preliminary study on the effect of **1** on sarcoplasmic reticulum (SR) Ca^{2+}-ATPase and survival of human lung adenocarcinoma cancer A_{549} cells has also been carried out.

Introduction

Since the discovery[1] of [60]fullerene and its large scale preparation,[2] more and more attention has been paid to the introduction of biologically active groups on to fullerenes because of its interesting physical and biological properties.[3] Steroids (sterols, such as cholesterol) are the main component of biomembranes and therefore we anticipated that coupling a fullerene with a steroid may change the physicochemical properties of the fullerene, improve its solubility and biocompatibility, and facilitate further studies on membrane–drug interactions and membrane-related processes such as signal transduction and solute transport *etc.* To the best of our knowledge, among the multitude of fullerene derivatives that have been published recently, only a very few examples of fullerenes with a steroid moiety have been reported.[4] Thus, we wish to report a synthesis of a new steroid-linked fullerene **1** from a steroidal enol-diene (Fig. 1) and the preliminary results of the effect of target molecule **1** on SR Ca^{2+}-ATPase, and survival of human lung adenocarcinoma cancer A_{549} cells.

Due to the pronounced dienophilic character of its [6–6] electron-deficient double bond, the Diels–Alder reaction has been demonstrated to be one of the most successful methods for the functionalization of fullerene.[3c,5] Accordingly, our synthesis of target molecule **1** used the Diels–Alder reaction of C_{60} with relatively sterically hindered silyloxydienes **2** and **3** (Fig. 1) as a key step.



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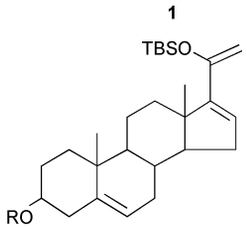
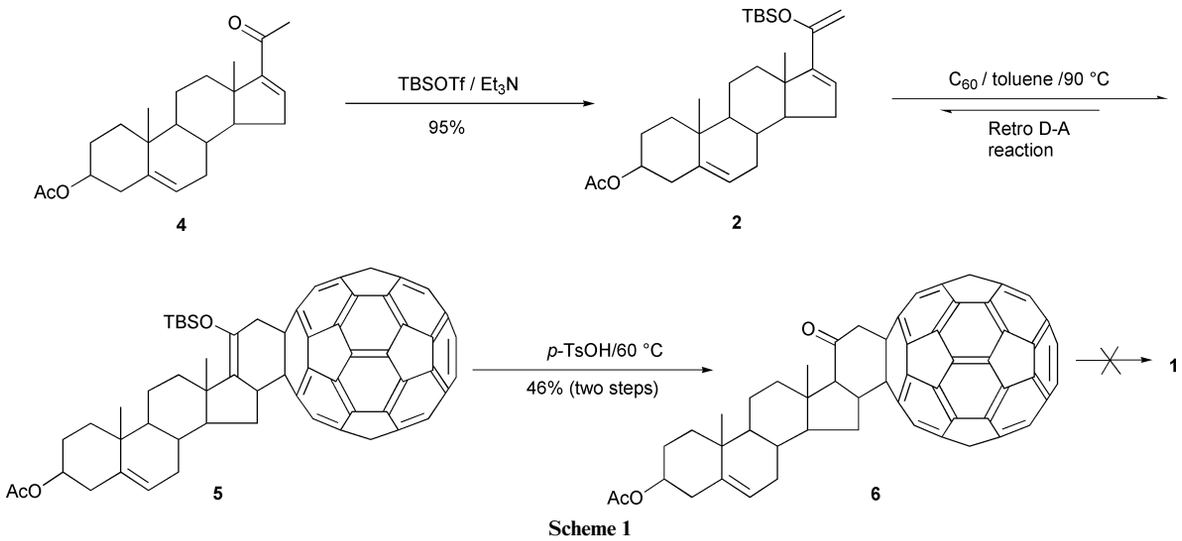


2 R=Ac
3 R=TBS

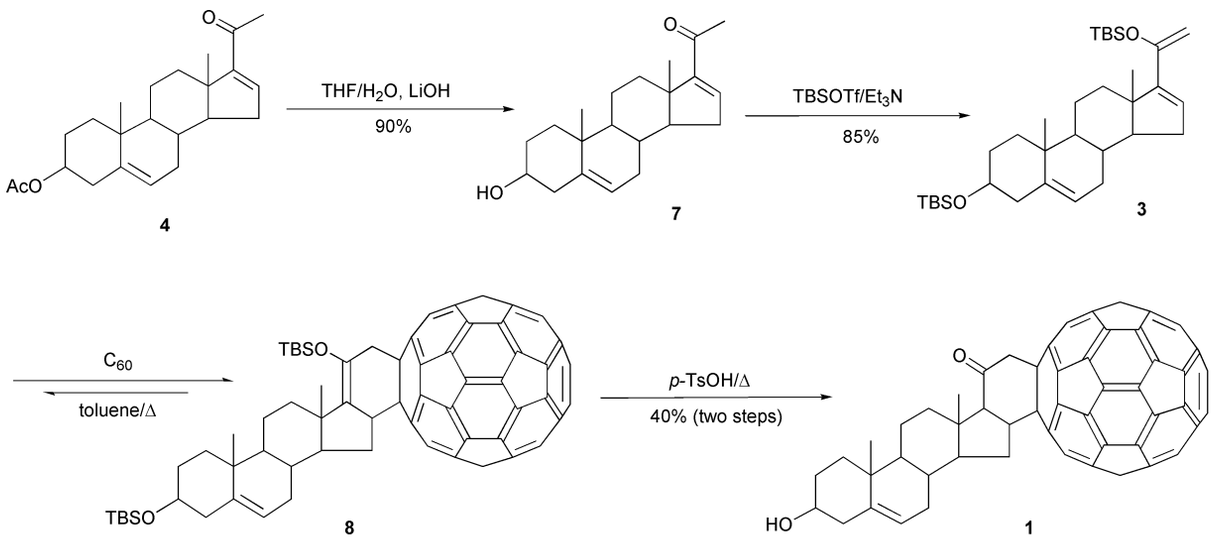
Fig. 1

Results and discussion

We began with examination of the cycloaddition of C_{60} to diene **2** (Scheme 1), which can be readily prepared from the available 3β-acetoxypregna-5,16-dien-20-one (**4**) (95% isolated yield) by treatment with *tert*-butyldimethylsilyl triflate (TBSOTf) and triethylamine. The following Diels–Alder reaction with C_{60} was carried out at 90 °C (at lower temperature the reaction did not occur at all) in toluene under a nitrogen atmosphere. TLC [silica gel, petroleum ether (PE)–CH_2Cl_2 = 2 : 1, R_f = 0.8] analysis showed the expected cycloadduct **5** was formed quickly and the reaction was completed within 4 h. A longer reaction time did not improve the yield of the product. However, all attempts to separate the adduct **5** by column chromatography on silica gel were unsuccessful due to the undesired retro Diels–Alder reaction.[3c,6] Therefore, the crude silyl enol ether intermediate **5** was converted directly to the corresponding stable ketone **6** by treatment with *p*-TsOH at 60 °C for 10 h followed by chromatography, as a brown solid, in 27% yield (over two steps, or 46% yield based on consumed C_{60}). It was noteworthy that only one stereoisomer **6** was obtained (the other stereoisomer was formed only in trace amounts and could not be identified clearly). The excellent diastereoselectivity was attributed to the induction of the substrate's chirality. The structure of product **6** was clearly established by spectroscopic means. Thus, both the ESI mass spectrum, [molecular-ion peak at *m*/*z* 1077 $[M+H]^+$ for $C_{83}H_{32}O_3$], and the MALDI-TOF spectrum of compound **6** [molecular-ion peak at *m*/*z* 1077 $[M+H]^+$ together with a base peak at *m*/*z* 720 due to the fragment C_{60}], corresponded with the proposed structure of compound **6**. In the ^1H NMR spectrum, owing to the effect of C_{60}, there was a significant difference in the chemical shift of the two 21-CH_2 geminal protons, which appeared as doublets downfield (δ_H 4.69 and 4.08, J = 16.8 Hz, geminal coupling) compared with the normal methylene protons. The configuration of **6** (Fig. 2) was confirmed by a large coupling constant $J_{16,17}$ = 12.2 Hz (*trans* coupling), and the nuclear Overhauser effects (NOE) between 16-H/18-CH_3, 16-H/



Scheme 1



Scheme 2

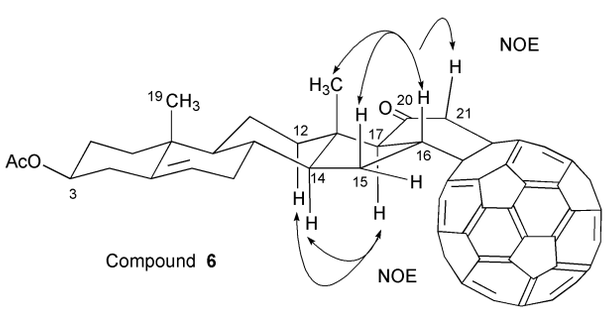


Fig. 2

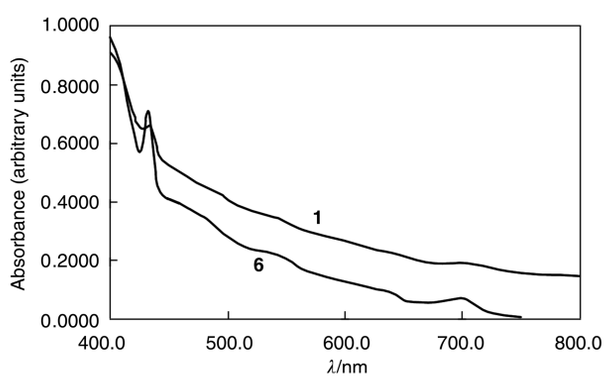


Fig. 3 UV-Vis absorption spectra of compound **1** and **6** in dichloromethane.

15β-H, 16-H/21β-H, 17-H/14-H and 17-H/12α-H. Further evidence was obtained from ^{13}C NMR, DEPT, ^{1}H–^{1}H COSY, 2D heteronuclear multiple quantum-filtered coherence (HMQC) NMR, FT-IR, UV-Vis, and CD spectra (see Experimental).

In comparison with the structure of cholesterol, the hydroxy group at C-3 in fullerene-steroid **6** is present in the protected form. Unfortunately, all efforts to remove the protective group under weakly basic conditions (such as $KHCO_3$–EtOH–CH_2Cl_2 and K_2CO_3–EtOH–CH_2Cl_2) failed. Under more basic conditions (LiOH–THF–H_2O), the product mixture was very complicated as shown by TLC (PE–CH_2Cl_2 = 1:9). We attempted the reaction under acidic conditions (acidic ion-exchange resin Amberlyst 15). The result was also disappointing. Therefore, we turned to an alternative route to finish the synthesis (see Scheme 2).

Steroid **4** was readily converted into **7** in 90% yield by treatment with LiOH. In a similar reaction, the desired silyloxydiene **3** was obtained in 85% isolated yield from **7** by reaction with *tert*-butyldimethylsilyl triflate (2.5 eq.). As expected, the target molecule **1** was successfully obtained in 30% yield (over two steps, or 40% yield based on consumed C_{60}) by reaction of C_{60} with **3** in toluene at 90 °C for 2 h, followed by hydrolysis of the silyl enol ether and deprotecting of the hydroxy group at C-3 using *p*-TsOH in one pot. Compound **1** was fully characterized by ^{1}H NMR, ^{1}H–^{1}H COSY, ^{13}C NMR, 2D heteronuclear multiple quantum-filtered coherence (HMQC) NMR, UV-Vis,

Table 1 Effect of steroid-C_{60} (**1**) on the ATP hydrolysis activity of Ca^{2+}-ATPase

Steroid-C_{60} content	0	1%	3%	5%
Ca^{2+}-ATPase ATP hydrolysis activity	3.49 ± 0.01	1.47 ± 0.02	1.43 ± 0.04	1.12 ± 0.06
Activity ratio (%)	100	42	41.0	32

Fig. 4 CD spectra of compound **1** (thin line) and **6** (thick line) in dichloromethane (4×10^{-4} mol dm $^{-3}$, 10 mm cell).

CD and FT-IR. All spectra are consistent with the proposed structure.

The UV-Vis absorption spectra of **1** and **6** (Fig. 3) over 400–800 nm showed a similar absorption pattern as those reported in the literature.[4i] There is a sharp absorption in the spectrum of compound **6** with $\lambda_{max} = 432$ nm, which is very characteristic for the spectra of the mono-adducts from addition across a 6–6 bond of fullerene.[7] The corresponding band in the spectrum of compound **1** is weaker.

Recently, the chiroptical properties of chiral fullerene derivatives have been studied by CD spectroscopy.[8] The chiral fullerene-steroids **1** and **6** obtained here are also CD-active, with typical Cotton effects (Fig. 4). The CD spectra of **6** and **1** are similar in shape except for two differences: i) the Cotton effects in **6** are stronger than those in **1**, and ii) there is a weak positive Cotton effect at 456 nm ($\Delta\varepsilon = +0.19$) in the spectrum of **6** which is not observed for **1**. According to the sector rule for C_{60} derivatives reported by Wilson,[9] the sign of the Cotton effect at 430 nm can be used to determine the absolute configuration of attached groups. Clearly, the CD spectra of both fullerene-steroids show similar negative Cotton effects near 430 nm, and the negative sign is consistent with the sector rule model,[9] from which the proposed absolute configuration of compound **6** and **1** (see Fig. 2) can be further confirmed.

After the target molecule steroid-C_{60} (**1**) had been obtained, the study of its cytotoxic effects both at subcellular and cellular level was carried out. In the former case the effect on the reconstituted sarcoplasmic reticulum membrane transmembrane Ca^{2+}-ATPase (SR Ca^{2+}-ATPase) in soybean phospholipid liposomes was examined. The preliminary results in Table 1 and Fig. 5 show that steroid-C_{60} (**1**) obviously decreased both the ATP hydrolysis and Ca^{2+} uptake activity of Ca^{2+}-ATPase and the inhibitions were concentration-dependent. It is well known that cholesterol can decrease the lipid fluidity of biomembranes. The lower activity of membrane-bound Ca^{2+}-ATPase may result from a change in the physical state of the lipid induced by steroid-C_{60}. However, here the C_{60} moiety may increase such an effect of cholesterol owing to steric hindrance. Moreover, the effect of **1** on the survival of human lung adenocarcinoma A_{549} cells was also studied. The cell line was maintained in cultures in complete medium (Eagle's balanced salt solution with 10% heat-inactivated fetal bovine serum, 4 mM

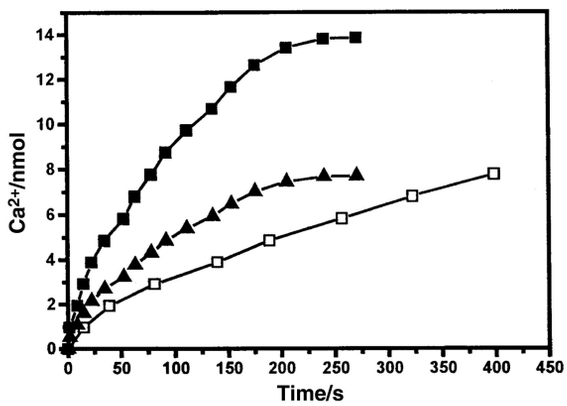


Fig. 5 Effect of steroid-C_{60} (**1**) on the Ca^{2+} uptake activity of reconstituted Ca^{2+}-ATPase in soybean phospholipid liposomes (SPL): ■ soybean phospholipid liposomes (SPL); ▲ SPL containing 1% Steroid-C_{60}; □ SPL containing 3% Steroid-C_{60}.

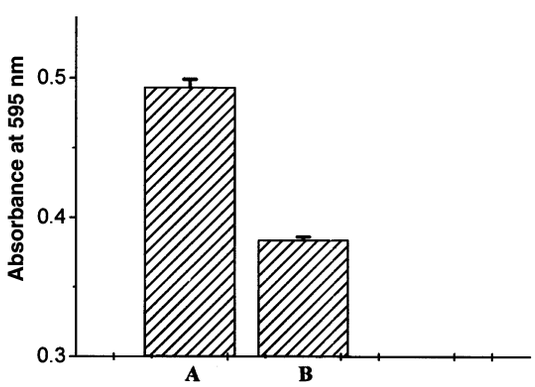


Fig. 6 Effect of steroid-C_{60} (**1**) on survival of human lung adenocarcinoma cancer A_{549} cells.

glutamate, 50 units ml^{-1} penicillin G and 50 units ml^{-1} streptomycin) at 37 °C in the dark, in 5% CO_2. Cells were resuspended to 1×10^6 cells ml^{-1}, and 100 μL was plated into a 96-well plate. The survival was evaluated after 48 h of incubation by the calorimetric method using the reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to MTT formazan, which was determined by measurement of the absorbance at 595 nm. Results obtained clearly showed that the survival of A_{549} cells following treatment with steroid-C_{60} (**1**) was obviously decreased (Fig. 6). Eukaryotic cells are rich in membranous structures. It is tentatively suggested that the steroid-linked fullerene mediated changes in physical state of membrane lipid could result in abnormality of cell functions and hence decrease the survival of A_{549} cells. Generally, the susceptibility of A_{549} cells to anticancer drugs is quite low. So, the mechanism of such steroid-C_{60} effect deserves further investigation. Further studies are still in progress.

Conclusions

In summary, we have carried out the synthesis of a steroid-linked fullerene based on the Diels–Alder reaction between C_{60} and silyloxydienes **2** and **3**, followed by hydrolysis of the silyl enol ether under acidic conditions. The fullerene steroids **6** and **1** were fully characterized by spectral analyses. Compound **1** with the synthetically versatile ketone and hydroxy function-

alities permits the preparation of a variety of other C_{60}-derivatives with interesting applications. Results of a preliminary assay show that compound **1** can inhibit the reconstituted SR Ca^{2+}-ATPase in soybean phospholipid liposomes and affect the survival of A_{549} cells.

Experimental

Synthesis and structure

General. The ^{1}H NMR, ^{13}C NMR, ^{1}H–^{1}H COSY, ^{1}H NOESY and HMQC spectra were recorded on a Bruker AMX-600 operating at 600 MHz for ^{1}H (some ^{1}H spectra were recorded on a Bruker AMX 300) and 150 MHz for ^{13}C with TMS as the internal standard, respectively. IR spectra were recorded on a Bruker FT instrument. Mass spectra were taken on a VG Quattro MS/MS or an HP5989A instrument. HRMS (EI) spectra were obtained on a Finnigan Mat 8430 mass spectrometer. The UV-Vis spectra were obtained with a Shimadzu UV-240 spectrometer. Optical rotations were measured on a Perkin-Elmer 241 MC polarimeter. Flash column chromatography was performed on silica gel H (10–40 μm) (or Al_2O_3) with a petroleum ether–dichloromethane or petroleum ether–ethyl acetate system as eluant. Microanalyses were carried out in the Microanalytical Laboratory at Shanghai Institute of Organic Chemistry.

3β-Acetoxy-20-(*tert*-butyldimethylsilyl)oxypregna-5,16,20-triene (2).[10] To an ice-cooled solution of **4** (712 mg, 2 mmol) in anhydrous ether (30 ml) was added dropwise triethylamine (332 μl, 2.4 mmol) and *tert*-butyldimethylsilyl triflate (0.5 ml, 2.2 mmol). After stirring at 0 °C for 15 min and then at room temperature for 30 min, the mixture was concentrated under reduced pressure, then passed through a pad of Al_2O_3 (petroleum ether as the eluant) to give compound **2** as a white solid (1.25 g, 95% yield): ^{1}H NMR (CD_3COCD_3; 300 MHz): δ 6.01 (1 H, dd, J = 3.0, 2.0 Hz), 5.40 (1 H, dd, J = 4.1, 1.3 Hz), 4.50 (1 H, m), 4.48 (1 H, s), 4.29 (1 H, s), 2.35–1.0 (17 H, m), 1.97 (3 H, s), 1.08 (3 H, s), 0.97 (3 H, s), 0.95 (9 H, s), 0.16 (3 H, s), 0.15 (3 H, s). EIMS (*m*/*z*, %): 470 (M^+), 455 (55.54), 399 (100.00), 339 (42.23), 235 (3.54), 75 (56.73). IR (KBr): 3060, 2932, 1736, 1640, 1571, 1249, 1039, 1004, 832 cm^{-1}.

Steroid-C_{60} (6). A mixture of C_{60} (144 mg, 0.2 mmol) and silyloxydiene **2** (141 mg, 0.3 mmol) was dissolved in 70 mL of anhydrous toluene under nitrogen, and the solution was heated at 90 °C for 4 h. Then the solution was cooled to room temperature, *p*-TsOH (50 mg, 1.5 eq.) was added, and the stirring was continued for 10 h at 60 °C. The solvent was then evaporated under reduced pressure, and the residue was chromatographed on silica gel (eluting with first toluene then 2 : 1 petroleum ether–dichloromethane) to afford C_{60} (60 mg, 42%), and compound **6** (58 mg, 27%, 46% based on consumed C_{60}). ^{1}H NMR ($CDCl_3$; 600 MHz): δ 5.30 (1 H, d, J = 3.6 Hz), 4.69 (1 H, d, J = 16.8 Hz, 21-H), 4.60 (1 H, m, 3-H), 4.56 (1 H, dt, J = 4.2, 12.6 Hz, 16-H), 4.08 (1 H, d, J = 16.8 Hz, 21-H), 3.58 (1 H, d, J = 12.6 Hz, 17-H), 2.79 (1 H, m), 2.02 (3 H, s), 2.59–1.14 (16 H, m), 1.27 (3 H, s), 1.11 (3 H, s). ^{13}C NMR ($CDCl_3$; 75 MHz): δ 208.69 (CO), 170.44 (CH_3CO), 156.44, 156.29, 154.59, 151.71, 147.73, 146.49, 146.34, 145.84, 145.58, 145.00, 144.61, 143.27, 142.68, 142.22, 141.87, 141.59, 140.01, 139.37, 136.81, 135.56, 135.18, 133.86, 121.84, 73.74 (C-3), 67.60 (sp^3-C for C_{60}), 66.97 (C-17), 64.36 (sp^3-C for C_{60}), 56.69, 54.31 (C-21), 49.92, 46.73 (C-16), 43.20, 38.06, 36.96, 36.71, 31.53 (2 C), 27.69, 26.89, 26.40, 21.39 (CH_3, OAc), 20.37, 19.38 (CH_3), 13.59 (CH_3). ESI MS: 1077 (M^+ + 1 for $C_{83}H_{32}O_3$). MALDI-TOF MS: 1077 (M^+ + 1, 24), 720 (C_{60} fragment, 100). IR (KBr): 2924, 2850, 1724, 1239, 1031, 751, 527 cm^{-1}. UV-Vis: 700.0, 432.0, 310.0, 256, 228, 222, 218, 212, 208, 204 nm. CD spectrum (dichloromethane) λ_{max} ($\Delta\varepsilon$) 674 (+0.66), 602 (+0.82), 456 (+0.19), 432 (−0.02), 285 (+0.14) nm.

3,20-Bis(*tert*-butyldimethylsilyl)oxypregna-5,16,20-triene (3). Compound **3** was obtained in 85% yield by a similar procedure to the synthesis of compound **2**, but the amount of *tert*-butyldimethylsilyl triflate was changed to 2.5 eq. ^{1}H NMR ($CDCl_3$; 300 MHz): δ 6.01 (1 H, dd, J = 3.0, 1.9 Hz), 5.34 (1 H, m), 4.45 (1 H, s), 4.28 (1 H, s), 3.49 (1 H, dt, J = 10.7, 4.6 Hz), 2.30–1.35 (17 H, m), 1.03 (3 H, s), 0.98 (9 H, s), 0.96 (3 H, s), 0.93 (9 H, s), 0.15 (6 H, s), 0.07 (6 H, s). EIMS (*m*/*z*, %): 542 (M^+, 4.18), 527 (30.94), 486 (35.32), 429 (6.72), 352 (10.71), 249 (6.52), 141 (11.05), 75 (100.00). IR (KBr): 2957, 2930, 1670, 1615, 1472, 1253, 1093, 1043, 836, 778 cm^{-1}. HR EIMS calcd. for $C_{33}H_{58}O_2Si_2$ 342.39754, found 342.39583.

Steroid-C_{60} (1). A mixture of C_{60} (100 mg, 0.14 mmol) and silyloxydiene **3** (100 mg, 0.18 mmol) was dissolved in 60 mL of toluene under nitrogen, and the solution was heated at 90 °C for 2 h. Then the solution was cooled to room temperature, *p*-TsOH (40 mg, 1.5 eq.) was added, and the stirring was continued for 10 h at 60 °C. The solvent was then evaporated under reduced pressure, and the residue was chromatographed on silica gel (eluting with first toluene then 1 : 9 petroleum ether–dichloromethane) to afford C_{60} (35 mg, 35%), and compound **1** (43 mg, 30%, 40% based on consumed C_{60}). ^{1}H NMR ($CDCl_3$; 300 MHz): δ 5.34 (1 H, d, J = 3.5 Hz), 4.70 (1 H, d, J = 17.0 Hz, 21-H), 4.56 (1 H, dt, J = 4.2, 12.2 Hz, 16-H), 4.08 (1 H, d, J = 17.0 Hz, 21-H), 3.58 (1 H, d, J = 12.2 Hz, 17-H), 3.52 (1 H, m, 3-H), 2.79 (1 H, m), 2.6–1.2 (16 H, m), 1.29 (3 H, s), 1.12 (3 H, s). ^{13}C NMR ($CDCl_3$, 150 MHz): δ 208.73 (CO), 156.52, 156.36, 154.64, 151.80, 147.78, 146.64, 146.59, 146.55, 146.40, 146.37, 145.90, 145.86, 145,63, 145.48, 145.39, 145.05, 144.97, 144.76, 144.64, 144.59, 144.49, 143.31, 142.87, 142.72, 142.33, 142.26, 142.23, 142.16, 141.89, 141.77, 141.71, 141.64, 141.56, 141.12, 140.46, 140.28, 140.08, 139.35, 136.84, 135.56, 135.21, 133.88, 121.38, 71.39 (C-3), 67.69 (sp^3-C for C_{60}), 66.78 (C-17), 64.41 (sp^3-C for C_{60}), 56.77, 54.45 (C-21), 49.73, 46.96 (C-16), 43.25, 38.21, 36.61, 32.34, 31.67 (2 C), 30.97, 29.69, 26.45, 19.91, 19.05 (CH_3), 13.82 (CH_3). IR (KBr): 3411, 2932, 1720, 1431, 754, 527 cm^{-1}. CD spectrum (dichloromethane) λ_{max} ($\Delta\varepsilon$) 685 (+0.39), 616 (+0.41), 432 (−0.03), 283 (+0.05) nm.

Biological studies

Preparation of SR Ca^{2+}-ATPase. Rabbit SR was prepared according to MacLennan,[11] and SR Ca^{2+}-ATPase according to Coll and Murphy[12] with slight modification. The enzyme was purified to homogeneity on SDS-PAGE.

Preparation of proteoliposomes containing SR Ca^{2+}-ATPase. Preparation of soybean phospholipid proteoliposomes was based on the methods described by Gould *et al.*[13] and Tu and Yang.[14] The lipid–protein ratio was 100 : 1 (μmol–μmol).

ATP hydrolysis activity and Ca^{2+} uptake of Ca^{2+}-ATPase. The ATP hydrolysis activity was monitored at 30 °C by continuous spectrophotometry following the oxidation of NADH as described by Carafoli *et al.*[15] Ca^{2+} uptake activity was measured at 30 °C by following the decrease in the absorbance of arsenano III used as Ca^{2+} indicator (675–685 nm) in a Hitachi model 557 spectrophotometer as described by Gould *et al.*[13]

Cell culture and survival assay. Cell line A_{549} was from Dr Jing Gao (Institute of Biophysics, Chinese Academy of Sciences, Beijing). The cell line was maintained in cultures in complete medium (Eagle balanced salt solution with 10% heat-inactivated fetal bovine serum, 4 mM glutamate penicillin G and 50 units ml^{-1} streptomycin) at 37 °C in the dark, in 5% CO_2. The survival was evaluated after 48 h of incubation by the

calorimetric method using the reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to MTT formazan, which was determined by measurement of the absorbance at 595 nm. The steroid-C_{60} was dissolved in DMSO (dimethyl sulfoxide) and medium containing 0.15 mg ml^{-1} steroid-C_{60}.

Acknowledgements

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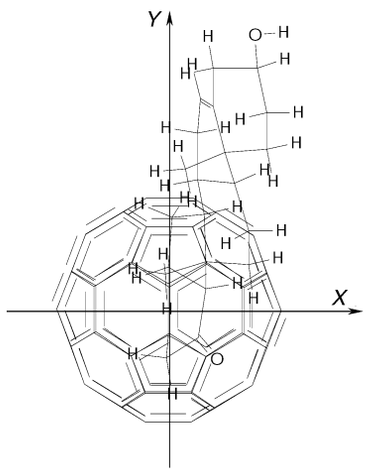

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