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Chromane derivatives of small aromatic molecules: Chemoenzymatic synthesis and growth inhibitory activity on human tumor cell line LoVo WT

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ABSTRACT

Aromatic substrates tyrosol (*p*-hydroxyphenylethanol) and 2,6-dihydroxynaphthalene (2,6-DHN) were converted into chromane derivatives by means of chemoenzymatic reactions catalyzed by the aromatic prenyltransferase of bacterial origin NovQ, using dimethylallyl bromide as allylic substrate instead of the natural isoprenyl pyrophosphate substrate. Stereoselective prenylation occurred in *o*-position with respect to the phenol hydroxyl in both compounds. Prenylated derivatives were readily converted into chromane products via a selective 6-*endo*-trig cyclization involving the oxygen atom from the phenol moiety and the double bond of the prenyl substituent, a process catalyzed by $FeCl_3$. These findings set up the basis of a most convenient two-step, one-pot process which allows for easy recovery of the chromane products in high yields. The chromane derivatives thus obtained were tested for cytotoxicity and pro-apoptotic activity using LoVo WT cells, a line of human colon adenocarcinoma.

1. Introduction

Prenylated aromatic compounds exhibit a wide spectrum of pharmacological effects as anti-microbial activity, pro-apoptotic activity, anti-proliferative activity against cancer cell lines, etc.[1–6]

Among the rich variety of natural prenylated molecules, chromane and chromene derivatives, with an extra pyrano or dihydropyrano ring, represent a family of compounds endowed with most interesting properties.[7] All these molecules are generally characterized by low cellular toxicity and good membrane permeability, properties that make them ideal drug template compounds. Some of these molecules have been shown to be able to inhibit mycobacterial growth,[8] to be promising therapeutic agents for AIDS[9] and to posses antitumoral activity.[10–12] Recently, the use of chromane derivatives as therapeutic agents in the treatment of cancer and cell proliferative disorders has also been reported.[13,14] Based on these data, the development of novel chromane-like molecules with potentially high biological activity for the design of new drugs or as molecular building blocks for chemical synthesis is a compelling target for pharmaceutical applications.

So far, most of such active compounds have been obtained as natural products via direct isolation mainly from higher plants.[15,16] However, prenylated compounds often exist at trace levels in natural sources. Thus, chemical or enzymatic synthesis of large amount of prenylated aromatic products is highly desirable.

The advances in the research on aromatic prenylated compounds led to the identification of a novel class of prenyl transferases (PTases) from *streptomycetes*, which are able to transfer dimethylallyl or geranyl chains to phenyl- and naphthyl-rings. Among these enzymes, the best characterized is *Streptomyces* sp. prenyltransferase Orf2 (recently renamed NphB), involved in the biosynthesis of isoprenoid-polyketide antibiotics. NphB is capable of transferring isoprenoid chains to a broad range of polycyclic aromatic molecules.[17,18] This enzyme has been tested for the selective geranylation towards flaviolin, flavonoids and dihydroxynaphthalenes. The less characterized PTases CloQ from *Streptomyces roseochromogenes* and NovQ from *Streptomyces spheroids*, instead, are involved in the specific prenylation of monocyclic aromatic compounds like *p*-hydroxyphenylpyruvate and *p*-hydroxybenzaldehyde.[19] Although all these enzymes are able to work in vitro in the presence of these aromatic molecules and isoprenyl pyrophosphate, the reaction yields are rather low, partially due to the spontaneous dephosphorylation of dimethylallyl and geranyl pyrophosphates in water. Moreover, the high cost of pyrophosphoriled substrates makes the process impractical for the large scale synthesis of prenylated aromatic products.

In the present paper we report the overexpression of the NovQ prenyltransferase in *Escherichia coli* and the construction and overexpression of a double mutant, which is able to accommodate

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larger aromatic substrates. Both the wild type protein and the mutant have been tested by using the less expensive and more stable dimethylallyl bromide as aliphatic substrate, and tyrosol and 2,6-DHN as aromatic substrates. Moreover, in order to modulate and improve the activity of the prenylated compounds obtained by the enzymatic reaction, we performed an iron-catalyzed cyclization leading to chromane derivatives. The cytotoxic properties of chromane derivatives have been evaluated against LoVo WT cultured cells of human colon adenocarcinoma.

2. Results and discussion

2.1. Enzymes

Aromatic prenyltransferases (PTAses) have been demonstrated to be useful as biocatalysts for the production of prenylated compounds both in vivo and in vitro. In particular, *Streptomyces* sp. prenyltransferase NphB showed broad specificity and good stereoselectivity in the prenylation of polycyclic aromatic molecules.[17,18]

In contrast, NovQ was shown specifically recognizes monocyclic aromatic compounds as substrate.[19] In order to broaden the substrate specificity of NovQ a set of mutants has been designed on the basis of a three-dimensional theoretical model obtained by homology modeling on the X-ray structures of NphB (Fig. 1). The topological positions R160 and Y233 appeared as the best candidates for mutagenesis studies in that these residues occupy the 'aromatic binding pocket' of the active site, located within the β-barrel (see Fig. 1, panel B). Thus, single and double mutants R160A, Y233A and R160A-Y233A have been designed cloned and overexpressed in *E. coli* under the control of an IPTG inducible promoter as soluble proteins of 34 kDa (about 100 mg soluble protein/l culture).

The catalytic activities of all four enzymes were tested against the aromatic substrates tyrosol and 2,6-DHN in the presence of dimethylallyl pyrophosphate, the natural substrates. The reaction products were obtained in moderate isolated yield (50–70%) and good selectivity with respect to blank reactions in the absence of enzymes, as shown by GC/MS analysis carried out at the end of the reaction (see Section 4). As a result, it was observed that whereas NovQ was the most active enzyme on tyrosol, the double mutant R160A-Y233A performed better on 2,6-DHN (data not shown). However, similar reaction yields were observed by using dimethylallyl bromide instead of the more expensive and less stable pyrophosphate. The experimental situation changed radically when using alcohols (ethanol, 2-metoxyethanol, isopropanol and isoamylic alcohol) as cosolvents to allow for better solubilization of the alkyl bromide. In particular, 20% isopropanol/phosphate buffer mixtures at pH 7.0 proved to be the best reaction medium for NovQ catalyzed prenylations. As a control, blank reactions in the absence of enzyme have also been performed and yielded a mixture of *o*- and *m*-prenylated compounds.

2.2. Chemoenzymatic synthesis

2.2.1. Chemoenzymatic synthesis of 2-(2,2-dimethyl-3,4-dihydro-2H-chromen-6-yl)ethanol (compound 1)

Tyrosol is efficiently prenylated by wt NovQ in isopropanol/buffer mixtures in the presence of prenylbromide. As demonstrated by GC/MS analysis performed on the reaction mixture tyrosol was converted into *o*-prenyl-tyrosol (**1**) after 12 h with a yield >80%. Mass spectrum of compound **1** derivatized with trimethylsilyl chloride showed a fragmentation pattern characterized by the molecular ion $m/z = 350$, which accounts for the presence of a trimethylsilyl-group on both the aliphatic and phenol hydroxyl groups. Similarly to what reported for metal-catalyzed chromane and chromene formation,[20] addition of catalytic amounts of $FeCl_3$ to the reaction mixture containing prenyl derivative **1** led to bicyclic compound **2**. The process most likely occurred via a selective 6-*endo*-trig cyclization involving the oxygen atom from the phenol moiety and the double bond of the prenyl substituent. As **2** is insoluble in aqueous solutions, the compound was easily recovered by centrifugation as a light brown powder and characterized by GC/MS, ^1H NMR and ^{13}C NMR. As shown by the GC/MS chromatogram, **2** has been obtained with a 98% purity and 78% isolated yield.

The derivatization of **2** with TMS chloride led to the substitution of only one silyl group on the aliphatic hydroxyl; the phenolic hydroxyl is unreactive as it is part of the chromane backbone.

2.2.2. Chemoenzymatic synthesis of 3,3-dimethyl-2,3-dihydro-1H-benzo[*f*]chrome-8-ol (compound 5) and 2,2,8,8-tetramethyl-2,3,4,8,9,10-hexahydrochromeno[6,5-*f*]chromene (compound 6)

NovQ R160A-Y233A was able to catalyze the prenylation of 2,6-dihydroxynaphthalene in the presence of dimethylallyl bromide (Scheme 1). Similarly to tyrosol, the prenylation reaction on 2,6-DHN occured on the *o*-position to the phenolic hydroxyls. However, the formation of two compounds is observed, the first (**3**) bearing one prenyl group on C1 and the second (**4**) bearing two prenyl groups on C1 and C5. These findings were confirmed by GC/MS analysis on TMS derivatives obtained after reaction of the crude mixture with trimethylsilyl chloride. Also in this case the addition of catalytic amounts of $FeCl_3$ led to the formation of monochromane **5** and dichromane **6** from compounds **3** and **4**, respectively. Chromane derivatives **5** and **6** were obtained in 30% yield each as yellow solids. The mixture of the two products could be easily recovered via centrifugation and filtration of the crude reaction mixture.

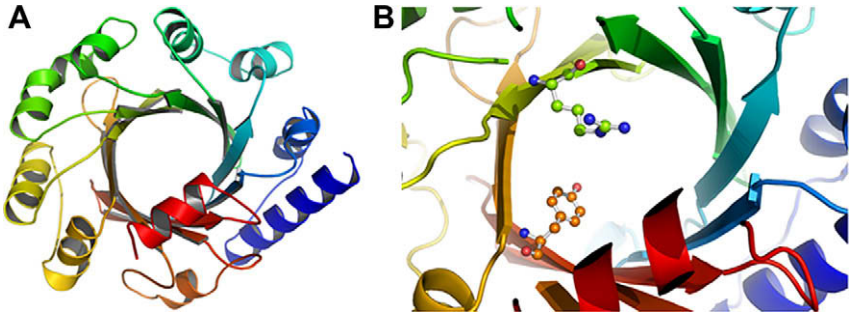
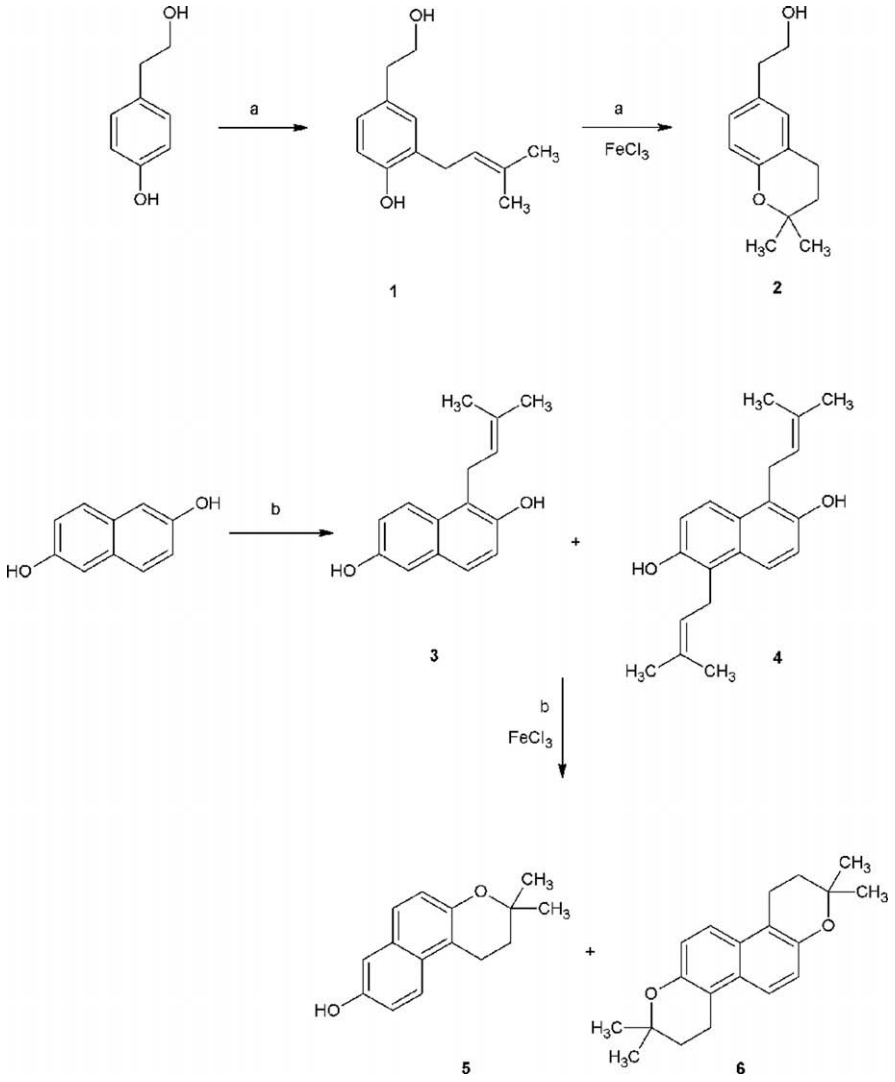


Figure 1. Structural model of NovQ. The overall fold is shown in panel A as a ribbon diagram. A detail of the active site is shown in panel B. R160 and Y233 residues subjected to mutations are indicated in ball-and-stick. The picture was generated with PYMOL.



Scheme 1. Reagents and conditions: (a) prenyl bromide, NovQ, isopropyl alcohol/phosphate buffer pH 7.5 (20% v/v), 16 h, 25 °C; (b) prenyl bromide, NovQ R160A-Y233A, isopropyl alcohol phosphate buffer pH 7.5 (20% v/v), 16 h, 25 °C.

2.3. Bioactivity

Compounds **2**, **5** and **6**, obtained as described before, were further purified by preparative HPLC and characterized by NMR and GC/MS spectrometry (see Section 4.6). All compounds, dissolved in DMSO, have been tested for their in vitro antineoplastic activity towards LoVo WT cells. All these molecules, whose internalization into the cells has been demonstrated by uptake kinetics at 24 and 48 h, have been evaluated for their cytotoxic activity.

As shown in Figure 2, compound **2** is inactive, while 2,6-DHN derivatives posses a moderate cytotoxicity after 24 h incubation. The observed effect is time and concentration dependent. The IC_{50} calculated after 24 or 48 h incubation time in the presence of **5** and **6** were, respectively, 34.5 and 257.6 μM (Table 1). In order to assess the ability of **5** and **6** to induce apoptosis, the hypodiploidy (i.e., loss of fragmented DNA) of LoVo WT cells has been evaluated by flow cytometry.[21] As apparent from the results reported in Table 1, 24 h exposure of the tested cell line to **5** and **6** has shown that only **5** has a pro-apoptotic effect and is able to induce a mean increase in the percentage of hyplodiploid cells. As a control, 2,6-DHN does not show cytotoxicity neither pro-apoptotic activity leading to the conclusion that only its chromane-like functionalization is able to confer the desired biological activity.

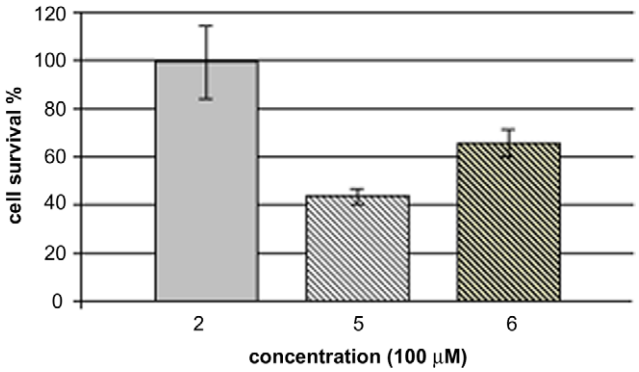


Figure 2. Cytotoxic effect of compounds **2**, **5** and **6** in LoVo WT cells. Cell viability was determined by the MTS assay and the percentage of cell viability was calculated as a ratio of drug-treated cells and control cells (treated with 0.2% DMSO only). The data are presented as the mean ± SD of eight independent experiments.

3. Conclusion

The results of the present investigation indicate that aromatic PTAase enzymes can be conveniently used in biotransformation

Table 1
IC_{50} values (mM) of compounds **5** and **6**. IC_{50} values (μmol/l) were determined in LoVo WT cells by MTS assay after 24 or 48 h treatment

Compound	Incubation time (h)	IC_{50}
5	24	34.51
	48	27.35
6	24	257.6
	48	395.4

processes to yield chromane derivatives. In particular, two interesting results are highlighted within the proposed biotransformation. First, NovQ PTAase recognizes the easily affordable prenylbromide substrate as an alternative to the highly unstable and expensive natural substrate, isoprenyl pyrophosphate. Second, the *o*-prenyl derivative thus obtained can be easily converted into the chromane scaffold by addition of $FeCl_3$ within the original reaction mixture. These findings set up the basis of a most convenient two-step, one-pot process which allows for easy recovery of the chromane products in high yield. The present process paves the way to the development of variously functionalized derivatives of aromatic phenols through easy and efficient modifications that are expected to increase their bio-availability and bioactivity. Further chemoenzymatic methods will be planned to synthesize novel functionalized aromatic compounds starting from **2**, **5** and **6**, in order to create more soluble and highly active molecules.

4. Experimental

4.1. Proteins expression and purification

Synthetic genes coding for *Streptomyces spheroids* prenyltransferase NovQ have been constructed by GENEART (GmbH, Germany) with optimised *E. coli* codons. The gene, designed with NdeI/BamHI restriction sites, was cloned directly within a pET22-b expression vector (Novagen, Darmstadt, Germany) cut with the same restriction enzymes. Competent *E. coli* BL21 (DE3) cells were transformed with the ligation mixture and the colonies with the correct DNA insert were selected through PCR screening.

Site directed mutagenesis was carried out on the pET22-b-novQ plasmid with the Quikchange mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. The mutants obtained were sequenced and transformed in *E. coli* BL21 (DE3) cells.

E. coli cells were grown in Luria–Bertani medium containing 100 μg/ml ampicillin. Wild type NovQ, R160A, Y233A and R160A-Y233A mutants expressions were induced by adding 1 mM IPTG to cells in exponential growth phase (OD_{600} = 0.6). After induction the cells were incubated overnight at 25 °C, pelleted, resuspended in a minimum volume of lysis buffer (50 mM Tris–HCl pH 8.0, 1.0 mM EDTA, 0.5 mM DTT, 100 mM NaCl, 10% glycerol, 1 mM PMSF) and sonicated until the supernatant was clear. After centrifugation, the supernatant was dialyzed versus 20 mM phosphate buffer pH 7.0, loaded on a DEAE–cellulose column (Whatman International Ltd, Maidstone, England) equilibrated with the same buffer and eluted with a NaCl linear gradient (0–0.5 M).

4.2. Model building of NovQ

Molecular model of NovQ was built as follows. Orf2, whose crystallographic structure is available,[17] was chosen as main template, from which all conserved main-chain regions were retained.

Side-chains of conserved residues were imported from the main structural template, those of mutated residues from a library of side-chain conformations frequently observed in protein structures (rotamers). Assignment of secondary structure elements to known structures performed with the DSSP program[22] was downloaded from the PDB web site. Secondary structure predictions for NovQ were performed with the programs PROF[23], PSIPRED[24] and SAM-T02[25].

The SASA of known structures was calculated with the NACCESS program, and residues were defined as buried when the SASA values were ⩽20 Å. Prediction of solvent accessibility for NovQ was performed with the program PROF. The reliability of the prediction was considered to be 'low' and 'high' for values of expected prediction accuracy of 0–4 and 5–9, respectively. InsightII[26] and its Biopolymer module (Accelrys Inc.) were used to perform: (i) structure visualization and analysis; (ii) optimal structure superpositions and RMSD calculations; (iii) all the steps required for model building; (iv) main-chain dihedral angle, hydrogen bond and contact calculations. Two atoms were defined as being in contact if their distance is lower than or equal to 3.5 Å.

4.3. Prenylation assays

Catalytic activities were explored for the recombinant purified proteins NovQ and R160A-Y233A mutant, by using tyrosol and 2,6-DHN as aromatic substrates, and dimethylallyl bromide as cosubstrates. The reaction condition consisted of 5 mM aromatic substrate, 10 mM dimethylallyl bromide, 10 μM enzyme, isopropyl alcohol/50 mM phosphate pH 7.5 (20% v/v), in a final volume of 500 μl. After overnight incubation at room temperature, 100 μl of the reaction mix were extracted twice with 200 μl of diethyl ether, dried and analyzed by GC/MS and NMR.

4.4. Cyclization runs

Crude reaction mixtures containing **1** or **3** and **4** were treated with $FeCl_3$ (1:20, w/w with respect to the aromatic substrate). After 3 h the conversion of the starting substrates was complete. The products **2** or **5** and **6** were recovered as light brown solids by centrifugation and filtration. After being washed with 500 μl of ethyl alcohol the solids were dried under vacuum (30% isolated yield). Samples of **5** and **6** for NMR analysis were purified by means of preparative HPLC.

4.5. GC/MS and NMR analysis

Before GC/MS analysis the samples were derivatized with 50 μl of silylation reagent at 25 °C for 30 min. The derivatized samples were suspended in ethyl acetate and analyzed by GC/MS on a Agilent 6850A gas chromatograph coupled to a 5973N quadrupole mass selective detector (Agilent Technologies, Palo Alto, CA, USA). Gas-chromatographic separations were carried out on a Agilent HP-5MS fused-silica capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm). Injection mode: splitless at a temperature of 260 °C. Column temperature program: 70 °C (1 min) then to 280 °C at a rate of 10 °C/min and held for 15 min. The carrier gas was helium at a constant flow of 1.0 ml/min. The spectra were obtained in electron impact mode at 70 eV ionization energy and a mass range scan from m/z 30 to 500; ion source temperature 280 °C, ion source vacuum 10^{-5} Torr.

NMR experiments were taken in $CDCl_3$ at room temperature on a Varian Unity Inova at 400 MHz and 296 K. Chemical shifts are expressed in δ (ppm) values relative to tetramethylsilane (TMS) as internal reference.

4.6. Spectral data of new compounds

4.6.1. 2-(2,2-Dimethylchroman-6-yl)ethanol

Compound **2** ^{1}H NMR spectrum (400 MHz, $CDCl_3$): δ 6.94 (1H, dd, J = 8, 2.5 Hz; H-7), 6.92 (1H, d, J = 2.5 Hz; H-5), 6.73 (1H, d,

J = 8 Hz; H-8), 3.81 (2H, t, J = 7 Hz, β-CH_2), 2.77, 2.76 (2 × 2H, 2 × t, J = 7 Hz; H_2-4, α-CH_2), 1.80 (2H, t, J = 7 Hz, H-3), 1.33 (6H, s, 2 × Me). ^{13}C NMR (100 MHz, $CDCl_3$): δ 153.7 (C-9), 129.8 (C-6), 128.3 (C-5), 126.1 (C-7), 120.0 (C-10), 111.6 (C-8), 73.1(C-2), 63.1 (β-CH_2), 39.5 (α-CH_2), 31.7 (C-3), 22.6 (C-4), 26.9 (2 × Me). GC/MS (EI, m/z): 206 (34.8%), 175 (100%), 151 (25.0%), 133 (15.1%), 91 (15.4%).

4.6.2. 3,3-Dimethyl-2,3-dihydro-1H-benzo[*f*]chrome-8-ol

Compound **5** ^{1}H NMR spectrum (400 MHz, $CDCl_3$): δ 7.74 (1H, d, J = 8 Hz, H-9), 7.46 (1H, d, J = 8 Hz, H-5), 7.13 (1H, dd, J = 8 and 2.5 Hz, H-6), 7.12 (1H, d, J = 2.5 Hz, H-8), 7.00 (1H, d, J = 8, H-10), 3.00 (2H, t, J = 7 Hz, H_2-3), 1.96 (2H, t, J = 7 Hz, H_2-4), 1.37 (6H, s, 2 × Me). ^{13}C NMR (100 MHz, $CDCl_3$): δ 154.2 (C-7), 151.8 (C-10a), 130.6 (C-8a), 127.8 (C-5), 126.9 (C-4-b), 126.2 (C-6), 126.1 (C-9), 124.4 (C-8), 119.1 (C-10), 111.7 (C-4a), 75.1 (C-2), 33.1 (C-3), 26.9 (2 × Me), 20.6 (C-4). GC/MS (EI, m/z): 228 (77.3%), 173 (100%), 144 (22.2%), 115 (27.7%), 127 (10.6%).

4.6.3. 2,2,8,8-Tetramethyl-2,3,4,8,9,10-hexahydrochromeno[6,5-*f*]chromene

Compound **6** ^{1}H NMR spectrum (400 MHz, $CDCl_3$): δ 7.64 (2H, d, J = 8 Hz; H-5, H-11), 7.04 (2H, d, J = 8 Hz; H-12, H-6), 3.02 (4H, t, J = 7 Hz: H_2-3, H_2-9), 1.94 (4H, t, J = 7 Hz; H_2-4, H_2-10), 1.37 (12H, s; 4 × Me). ^{13}C NMR (100 MHz, $CDCl_3$): δ 154.3 (C-12a and C-6a), 128.7 (C-4b and C-10b), 126.1 (C-5 and C-11), 118.7 (C-12 and C-6), 112.3 (C-4a and C-10a), 75.1 (C-2 and C-8), 33.1 (C-3 and C-9), 27.0 (4 × Me), 20.6 (C-4 and C-10). GC/MS (EI, m/z): 296 (100%), 241 (84.1%), 185 (35.9%), 128 (16.6%), 115 (48.9%).

4.7. Cytotoxicity assay

The cell line utilized in this study is LoVo WT, an human colon adenocarcinoma, obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in D-MEM F12 supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin G, 50 μg/ml streptomycin sulfate, and maintained in humidified atmosphere with 5% CO_2 at 37 °C. Cell disassociation was obtained with 0.05% trypsin—0.02% EDTA.

Compounds **2**, **5** and **6** were dissolved in dimethyl sulfoxide (DMSO) to give a stock solution of 100 mM from which further dilutions in culture medium were prepared. The residual solubilizing DMSO concentration after dilution (0.3% or less) was not cytotoxic.

The cytotoxicity of these compounds was determined using the MTS assay.[27] Briefly, individual wells of a 96-well microtiter tissue culture plate were inoculated with 100 μl of the growth medium containing 1×10^4 cell/well. After 1 day incubation, the growth medium was removed and replaced with exposure medium (100 μl), with or without different concentrations of the test agents (from 0.1 μM to 300 μM). After a 1–3 day exposure of the cells to the test agents, 20 μl of MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3carboxymethoxyphenyl]-2-[4-sulphophenyl]-2H tetrazolium) and PMS (phenazine methosulfate), were added directly to the cell culture and incubated for 2 h at 37 °C in a humidified, 5% CO_2 atmosphere. The combined MTS/PMS solution was obtained with Cell-Titer 96®AQueous MTS Reagent Powder (Promega Corporation, Madison, WI, USA) and PMS (Sigma–Aldrich Chemie GmbH) dissolved in Dulbecco's phosphate buffered saline (DPBS). The absorbance of formazan (metabolite of MTS by viable cells) was measured at 490 nm. The absorbance value was used to calculate the surviving cell number. The IC_{50} value (concentration of a compound that is required to inhibit 50% of cell growth) was calculated with dose–response curves.

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