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Enzymatic transformation of flavonoids and terpenoids: Structural and functional diversity of the novel derivatives*

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Abstract: Flavonoids and terpenoids are promising candidates for use as functional foods and novel therapeutics due to their prominent biological activities. However, the use of several bioactive plant compounds is limited by low stability and solubility problems. This review underlines the feasibility of enzymatic synthesis of novel bioactive analogs of selected flavonoids (silybin, rutin) and terpenoids (perillyl alcohol, POH) in nonconventional reaction systems. The effect of various parameters on the enzymatic acylation and/or glucosylation of these phytochemicals was studied. Also, the role of the structure of the novel molecules in relation to their biological function was investigated in various cancer cell lines.

Keywords: anti-tumor activity; biocatalysis; flavonoids; phytochemicals; terpenoids.

INTRODUCTION

Phytochemicals, plant compounds with low toxicity and prominent biological actions, are promising candidates for uses in the food and pharmaceutical industries as functional foods and novel therapeutics [1,2]. Flavonoids and terpenoids comprise a very interesting class of phytochemicals as they have been proved effective in prevention and therapy of several cancer types in vitro and in vivo [3–7]. However, chemical heterogeneity among phytochemicals, even within the same class, justifies differences in their action mechanisms, bioavailability, and pharmacokinetic profiles, thus necessitating investigation of the structural characteristics that are essential for biological function [8,9]. Furthermore, the use of several bioactive plant compounds is limited by low stability and solubility problems; thus, there is a requirement of improving their physicochemical properties by introducing selective modifications [10]. In order to improve their properties and broaden the field of their applications, chemical, enzymatic, or chemo-enzymatic modifications of a number of natural compounds have been performed by several researchers. It has been shown that biocatalytic modifications are superior compared to conventional

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chemical methods because of the mild reaction conditions of temperature and pressure which are applied, as well as of the high catalytic efficiency and the regio-, stereo-, and enantioselectivity of the biocatalysts that result in pure products [11–14].

Glycosylation and acylation are the two biocatalytic reactions that have received particular attention in order to enhance the hydrophilic or -phobic character by adding sugar or fatty moieties, respectively [15–18]. In this paper, we describe the enzymatic regioselective modification of silybin, rutin, and perillyl alcohol (POH) in nonconventional media. Lipase was used to catalyze the esterification and transesterification of silybin and rutin with simple and unsaturated fatty acids and their vinyl and methyl esters in various low water content organic solvents. Furthermore, POH was glucosylated and/or acylated using various glucosidases and lipases in biphasic systems or pure organic solvents in order to synthesize novel analogs with a different degree of hydrophobicity. The influence of several reaction parameters was examined, and herein only the optimum conditions are presented. Finally, the structure–biological function relationships of the native molecules as well as the new analogs was evaluated concerning their anti-tumor activity using various cancer cell lines.

MATERIALS AND METHODS

Chemical and biological materials

Silybin (a roughly 1:1 mixture of two diastereoisomeric compounds, silybin A and silybin B), rutin (95 %), (*S*)-(–)-POH, α-pinene, myrcene, D-(+)-glucose, unsaturated fatty acids, fatty acids, and their vinyl or methyl esters were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) or Fluka Chemie GmbH (Buchs, Switzerland.), and were of the highest purity. Silica gel 60 F_{254} aluminum plates for thin layer chromatography (TLC) were purchased from Merck (Darmstadt, Germany). Molecular sieves (10–20 mesh beads, pore diameter 3 Å) were purchased from Fluka. All solvents used were of the highest purity LabScan Analytical Sciences (LabScan Ltd., Dublin, Ireland). Lipase B from *Candida antarctica* (*CALB*) immobilized on a macroporous acrylic resin (Novozym 435®) was a generous gift of Novozymes Novo Nordisk A/S (Bagsvaerd, Denmark). β-Glucosidase from almonds (1.18 U mg^{-1} of powder) was purchased from Fluka.

K562 cell line and Lewis lung carcinoma (LLC) cells were originally obtained from American Type Culture Collection (Manassas, VA). Tissue culture plastic ware was obtained from Corning-Costar (Corning, NY). Cell culture media, fetal calf serum (FCS), and antibiotics were obtained from Life Technologies (Paisley, UK) and Gibco (Grand Island, NY). MTT viability assay was obtained from Sigma (St. Louis, MO). VEGF ELISA Duo set kit was provided from R&D Systems (Minneapolis, MN). Bio-rad DC protein microassay was obtained from Bio-Rad (Hercules CA, USA).

Biotransformations of the natural compounds

Enzymatic acylation of flavonoids

Analytical-scale reactions of silybin esters synthesis were carried out in sealed round bottom flasks using acetone, *tert*-amyl alcohol, *tert*-butyl alcohol, or acetonitrile as reaction medium. Silybin and various acylation agents (fatty acids and corresponding vinyl or methyl esters) were solubilized in the organic solvent (10 ml) previously dehydrated with 3 Å molecular sieves. The acylation of silybin was performed in the presence of immobilized lipase (15 mg ml^{-1}, Novozym 435). In all cases studied, the flasks were incubated at 50 °C and stirred at 250 rpm. Rutin ester synthesis was carried out in sealed round-bottom flasks using acetone as reaction medium. Rutin (55 mg) was solubilized in acetone (9 ml) previously dehydrated with 3 Å molecular sieves. The acylation of rutin was performed in the presence of immobilized lipase (100 mg, Novozym 435). The acyl donor concentration was adjusted to have a flavonoid/acyl donor molar ratio of 1/5. The mixture was incubated at 50 °C and stirred at 175 rpm.

Enzymatic transformation of POH

Analytical-scale reactions of (*S*)-(–)-POH esters synthesis were carried out in sealed round-bottom flasks using different α-pinene/myrcene ratios or pure organic solvents as reaction medium. (*S*)-(–)-POH (167 mM) and various acylation agents (167 mM), (propionic, decanoic, or hexadecanoic acid) were solubilized in the solvent (5 ml). The acylation of (*S*)-(–)-POH was performed in the presence of immobilized lipase (40 mg, Novozym 435). In all cases studied, the flasks were incubated at 50 °C and stirred at 250 rpm.

Glucosylation reactions were carried out in sealed round-bottom flasks using a biphasic system of citrate/phosphate buffer and POH (5.6 M) as reaction medium. β-D-glucose solution (0.4 M) was solubilized in the biphasic system (5 ml). The glucosylation of (*S*)-(–)-POH was performed in the presence of β-glucosidase from almonds (0.6 mg l^{-1}). The mixture was incubated at regulated temperature and stirred at 220 rpm.

For the synthesis of perillyl glucoside fatty ester (PGL), pure perillyl glucoside (PG) (30 mM) was solubilized in *tert*-amyl alcohol (1 ml) previously dehydrated with 3 Å molecular sieves. Vinyl laurate (200 mM) was used as the acyl donor. Esterification reactions were performed in the presence of immobilized lipase (4 mg, Novozyme 435) at 50 °C and 1100 rpm.

Analytical methods

Quantitative and qualitative analysis of the reaction products

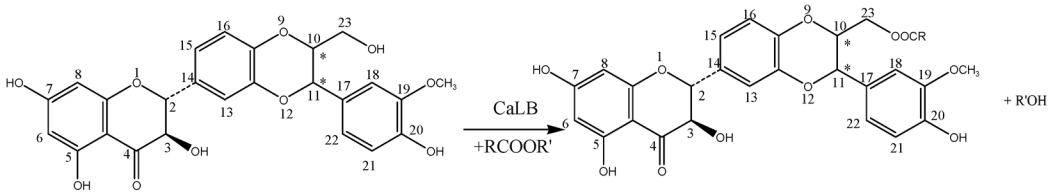
Qualitative analysis of silybin and rutin reaction mixtures was made by TLC on silica gel 60 F_{254} plates using a solvent mixture of petroleum ether/ethyl acetate (3/2) with 5 % acetic acid and chloroform/methanol/water (8/2/0.28), respectively. The plates were visualized and detected under UV light (254 nm). Quantitative analysis of silybin and rutin esters was monitored by high-performance liquid chromatography (HPLC) as described previously [15,16].

For the detection of PG and glucoside fatty ester, TLC analysis was performed on silica gel 60 F_{254} using a solvent mixture of chloroform/methanol (80/10) and (80/5), respectively. The compounds were visualized by spraying the plate with sulfuric acid/ethanol (10/90) solution and carbonization at 120 °C for 10 min. Concentrations of PG and D-(+)-glucose were determined by HPLC. HPLC analysis was carried out using a Waters 501 system equipped with a refractive index detector Waters 410. A Nucleosil NH_2 column (100-5, Macherey-Nagel) was used. An isocratic system of the eluent was used, having a flow rate of acetonitrile/water (90/10) 1 ml min^{-1}.

Purification and NMR characterization of silybin esters

Highly purified silybin esters were obtained by preparative HPLC [15]. The chemical structure of the purified monoester was determined by ^{1}H NMR spectroscopic analysis in dimethylsulfoxide (DMSO)-d_6 on a Varian Mercury-300 spectrometer (Varian GmbH NMR Instruments, GE).

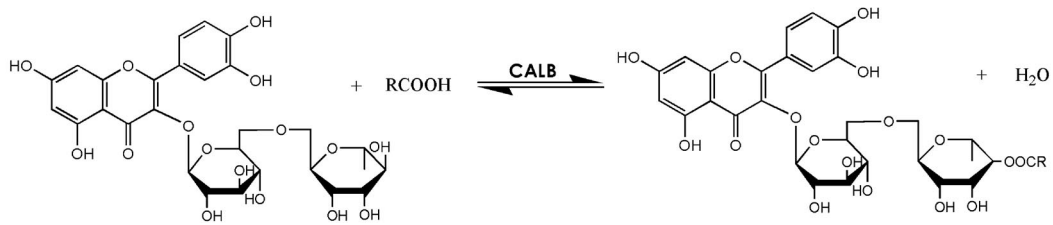
Silybin-23-*O*-butanoate (Scheme 1): ^{1}H NMR data (299.9 MHz, DMSO-d_6, 25 °C): 0.87 (3 H, t, *J* = 7.4, 3 × H-4'), 1.52 (2 H, m, 2 × H-3'), 2.29 (2 H, m, 2 × H-2'), 3.77 (3 H, s, *O*Me), 3.93 (1 H, ddd, *J* = 12.4, 4.9, H-23u), 4.13 (1 H, dd, *J* = 12.4, 2.7, H-23d), 4.51 (1 H, ddd, *J* = 8.0, 4.9, 2.7, H-10), 4.52 (1 H, ddd, *J* = 8.0, 4.9, 2.7, H-10), 4.59 (1 H, dm, *J* = 11.3, H-3), 4.92 (1 H, d, *J* = 8.0, H-11), 5.08 (1 H, d, *J* = 11.3, H-2), 5.80 (1 H, br s, 3-OH), 5.84 (1 H, d, *J* = 2.1, H-8), 5.85 (1 H, d, *J* = 2.1, H-8), 5.89 (1 H, d, *J* = 2.1, H-6), 6.80 (1 H, d, *J* = 8.0, H-21), 6.86 (1 H, dd, *J* = 8.0, 1.9, H-22), 6.98 (1 H, d, *J* = 8.3, H-16), 7.01 (1 H, d, *J* = 1.9, H-18), 7.02 (1 H, d, *J* = 1.9, H-18), 7.03 (1 H, dd, *J* = 8.3, 1.7, H-15), 7.03 (1 H, dd, *J* = 8.3, 1.7, H-15), 7.10 (1 H, d, *J* = 1.7, H-13), 7.11 (1 H, d, *J* = 1.7, H-13), 9.32 (1 H, s, 20-OH), 10.83 (1 H, s, 7-OH), 11.89 (1 H, s, 5-OH).



Scheme 1 Enzymatic acylation of silybin catalyzed by Novozyme 435.

Purification and NMR characterization of rutin esters

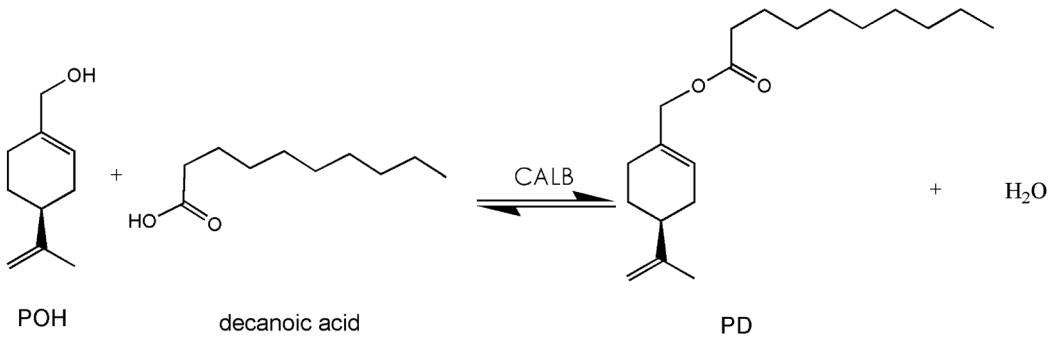
The rutin esters were isolated by flash point column chromatography [16]. The chemical structure of the purified rutin oleate (Scheme 2) was determined by ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) in methanol-d_6 using a Brucker AC 300 spectrometer. ^{1}H chemical shifts for rutin oleate were: δ (ppm) 8.5 (s, OH_5), 7.5 (m, H_2 and $H_{6'}$), 6.2 (d, $H_{5'}$), 6.1 (d, H_8), 5.4 (d, H_6), 5.2 (d, $H_{1''}$), 4.5 (t, $H_{6\ acylated}$), 3.8 (s, $H_{1'''}$), 3.7–3.2 (9H rhamnoglucosyl), 2.2–0.84 (fatty chain), 0.83 (d, CH_3 rhamnosyl). ^{13}C chemical shifts for rutin oleate were: δ (ppm) 177.8 (C4), 173.8 (C=O ester), 164.4 (C7), 161.5 (C5), 157.4 (C9 or C2), 156.9 (C2 or C9), 148.1 (C4'), 144.4 (C3'), 133.6 (C3), 129.2 (aliphatic chain), 121.8 (C6'), 121.5 (C1'), 115.8 (C5'), 114.4 (C2'), 104.0 (C10), 102.1 (C1''), 100.5 (C1'''), 98.3 (C6), 93.1 (C8), 76.5 (C3''), 75.3 (C5''), 74.1 (C2''), 73.4 (C4'''), 70.6 (C4''), 69.5 (C2'''), 68.7 (C3'''), 66.4 (C6''), 65.9 (C5'''), 33.5 (aliphatic chain), 31.5 (aliphatic chain), 29.2 (aliphatic chain), 29.0 (aliphatic chain), 28.9 (aliphatic chain), 28.8 (aliphatic chain), 28.6 (aliphatic chain), 26.5 (aliphatic chain), 24.4 (aliphatic chain), 22.1 (aliphatic chain), 16.0 (CH_3 rhamnose), 12.8 (CH_3 aliphatic chain).



Scheme 2 Lipase-catalyzed acylation of rutin with unsaturated fatty acids.

Purification and NMR characterization of perillyl decanoate

(*S*)-(–)-perillyl decanoate was isolated by column chromatography and preparative TLC [17]. The chemical structure of (*S*)-(–)-perillyl decanoate (Scheme 3) was determined by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (^{1}H NMR and ^{13}C NMR). The ^{13}C NMR chemical shifts for the decanoyl moiety in perillyl decanoate: δ 173.8 (C=O), 34.2 $CH_2(CH_2)_7CH_3$, 29.0–31.7 5 × CH_2, 24.3 $CH_2(CH_2)_6CH_3$, 22.4 CH_2CH_3, 13.9 CH_3. Ten carbon signals were observed for POH moiety: (δ 20.5, 26.2, 27.2, 30.3, 40.7, 68.1, 108.7, 125.6, 132.8, and 149.6 ppm). The signal at 68.1 ppm was assigned to the C-9 of the POH moiety. This signal exhibited a shift of 1.4 ppm in comparison with that of POH (δ 66.7), which suggests the presence of an ester bond [17].



Scheme 3 Lipase-catalyzed acylation of POH with decanoic acid.

Purification and NMR characterization of perillyl-glycoside and perillyl-glycoside monolauryl ester

The crude reaction product was purified by column chromatography on silica gel [18]. The chemical structures of perillyl β-D-glucopyranoside and monolauryl ester of perillyl glucopyranoside were confirmed by NMR analyses. The assignment of the peaks for the ester is presented below: ^{1}H NMR (CD_3OD) δ (ppm): 4.70 (s, 2H, H-9"), 1.72 (s, 3H, H-8"), 5.73 (s, 1H, H-7"), 2.15 (bs, 4H, H-5", H-6", H-4"a), 1.95 (m, 1H, H-4"b), 1.82 (m, 1H, H-3"a), 1.47 (m, 1H, H-3"b), 3.98 (d, 1H, H-1"a, J = 11.5 Hz), 4.13 (d, 1H, H-1"b, J = 11.5 Hz), 4.24 (d, 1H, H-1, J = 7.8 Hz), 3.17 (t, 1H, H-2, J = 7.9 Hz), 3.29 (t, 1H, H-3, J = 9.0 Hz), 3.26 (t, 1H, H-4, J = 9.1 Hz), 3.40 (m, 1H, H-5), 4.16 (q, 1H, H-6a, J = 6.2 Hz), 4.40 (dd, 1H, H-6b, J = 2.1 Hz, 11.8 Hz), 2.34 (t, 2H, H-2', J = 7.4 Hz), 1.61 (pentaplet, 2H, H-3', J = 7.4 Hz), 1.27 (bs, 16H, H-4'-11'), 0.88 (t, 3H, H-12', J = 7.2 Hz). ^{13}C-NMR (CD_3OD) δ (ppm): 149.5 (C-10"), 107.8 (C-9"), 19.5 (C-8"), 124.8 (C-7"), 26.1 (C-6"), 41.0 (C-5"), 30.3 (C-4"), 27.3 (C-3"), 134.1 (C-2"), 73.2 (C-1"), 101.5 (C-1), 73.6 (C-2), 76.5 (C-3), 70.3 (C-4), 73.8 (C-5), 63.2 (C-6), 173.9 (C-1'), 33.6 (C-2'), 24.7 (C-3'), 28.8–29.4 (C- 4'-9'), 31.7 (C-10'), 22.3 (C-11'), 13.0 (C-12'). NMR nuclear Overhauser effect (NOE) signal between H-1"a and H-1"b of POH with H-1 of D-(+)-glucose and HMBC signal between C-1 of D-(+)-glucose with H-1"a and H-1"b of POH provide evidence that the glucosylation took place at position 1 of the D-(+)-glucose. Additionally, the NOE signal between H-2' of the lauryl moiety with H-6a and H-6b of D-(+)-glucose and HMBC signal between C-1' of the lauryl moiety with H-6a and H-6b of D-(+)-glucose proves that the transesterification is performed at position 6 of the D-(+)-glucose (Scheme 5).

Biological assays

Cell culture

Human K562 lymphoblastoma and mouse LLC cells were maintained in RPMI 1640 and DMEM, respectively, supplemented with 10 % v/v FCS, L-glutamine, penicillin (10 U ml^{-1}), and streptomycin (100 mg ml^{-1}) in a humidified 37 °C incubator containing 5 % CO_2. Stock solutions of test compounds were prepared in DMSO or ethanol, and further dilutions were performed in culture media (final vehicle concentration did not exceed 0.1 % v/v).

Cell proliferation

Cells were plated in 96-well plates at 3–5 × 10^3 cells/well, 24 h later fresh media containing different concentrations of test compounds (25–100 μM of silybin and silybin esters, POH and POH derivatives (0.1–0.5 mM) or DMSO (0.1 % v/v) or ethanol vehicle) were added and cultures were further incubated for 24–72 h. At treatment completion, cell numbers were determined either by the MTT method [19] or by the CyQUANT cell proliferation assay kit as described previously [7]. Absorbance measurements were correlated with cell numbers by using a reference standard curve.

Vascular endothelial growth factor (VEGF) detection

K562 cells grown to confluence in 24-well plates (1×10^6 cells/well) were treated with fresh media containing different concentrations of test compounds (1–100 μM of rutin, rutin esters, and free fatty acids; 0.5 mM of POH) or vehicle (0.1 % DMSO) for 24–48 h. Conditioned media were then collected by centrifugation for VEGF detection, whereas cells were solubilized and total protein was measured. VEGF levels were measured using an ELISA kit detecting human VEGF according to the manufacturer's specifications. Results were normalized to total protein.

Data analysis and statistics.

All values represent mean ± SEM. The Student's or Mann–Whitney U tests were used to test for differences in the means between two groups and one-way analysis of variance (ANOVA) with Tukey post-hoc tests or the Kruskal–Wallis analysis were used to test for differences in the means between multiple groups, depending on the normality of data distribution. All p values are two-tailed; p values < 0.05 were considered significant. All statistical analyses were done using the Statistical Package for the Social Sciences v.13.0.0 (SPSS).

RESULTS AND DISCUSSION

The great interest for natural products, such as vitamins and plant phenolics (flavonoids and phenolic acids), stems from their biological properties which in addition to free radical scavenging activity include antibacterial, antiviral, immune-stimulating, antiallergic, antiinflammatory, antiatherogenic, anticarcinogenic, and estrogenic effects [20–22]. On account of these properties, natural products, especially phenolic antioxidants, can find numerous applications in pharmaceutical and cosmetic formulations as well as food additives [20,23]. Recently, several groups have worked on the modification of natural products, in order not only to modify their physicochemical properties but also to obtain analogs that can be used to study their structure–activity relationships. The modification of antioxidants can be achieved through chemical reactions, however, biocatalytic modification is preferred as it is more selective, requires milder conditions, enables the easier isolation of product, and is environmentally friendly [10,24]. The type of modification employed depends on the desired properties of synthesized derivatives. Therefore, in order to increase the hydrophilic character of natural antioxidants, specific sugar transferases and glycosidases have been employed in glycosilation reactions [25–27], while in order to increase the lipophilic character of these compounds hydrolytic enzymes, such as proteases and mostly lipases, have been employed. For enzymatic acylations of natural antioxidants, the combination of a hydrophilic and a hydrophobic substrate is a major concern. In this sense, the selection of a reaction medium that allows the solubilization of substrates is crucial [28]. Other factors that have to be considered for the selection of appropriate medium are: (i) to be able to maintain high enzyme activity and stability, (ii) to allow the recovery of reactants, (iii) to be compatible in terms of toxicity depending on the application (pharmaceuticals, cosmetics, foods) of the synthesized product [28–30]. In order to identify the proper reaction system for the enzymatic acylation of natural antioxidants, such as flavonoids, phenolic acids, and vitamins, several reaction media have been investigated. Recently, the use of ionic liquids (organic salts consisting of ions which remain liquid at or close to room temperature) has attracted increasing attention for the modification of various natural antioxidants [14,31–35]. However, in the majority of studies, organic solvents have been employed as reaction media. The use of organic solvents for the modification of natural antioxidants is reviewed in brief in this work.

Polyhydroxylated antioxidants, such as flavonoids, were interesting targets in order to investigate the behavior of enzymes with respect to the regioselectivity of acylation reactions and also to produce either novel derivatives or stable analogs. At this point, investigations were employing various enzymes. For instance, Danieli et al. (1990 or 1997) used subtilisin (protease from *Bacillus licheniformis*) to successfully acylate rutin, hesperidin, naringin, and quercetin with trifluoroethyl butanoate in pyridine [36], or immobilized lipase B from *C. antarctica* (Novozym 435) to acylate isoquercitrin, rutin, and

naringin with vinyl acetate in pyridine-acetone mixture [37]. Several lipases were used by Nakajima et al. (1999) in order to acylate flavonoid glucosides with activated vinyl esters of phenolic acids and among them *C. antarctica* lipase B and lipase from *Pseudomonas cepacia* were found to be suitable catalysts [38]. The same group (2000) was able to perform the direct acylation of flavonoid glucosides with phenolic acids using an enzyme solution from cultured cells of *Ipomoea batatas* [39]. At this early stage, chemoenzymatic approaches were also used for the acylation of flavonoids or chemically synthesized flavonoid glycosides with phenolic acids [40–43].

More recent studies focus on the parameters affecting the performance of enzymatic acylation of flavonoids. The lipase used is almost exclusively the immobilized lipase B from *C. antarctica*. Instead of toxic solvents, such as pyridine and mixtures with other solvents, Kontogianni et al. (2001, 2003) tested less toxic solvents (*tert*-butanol, acetone, tetrahydrofuran, chloroform, acetonitrile, and methanol) or solvent free media and longer acyl donors for the esterification of rutin and naringin [44,45]. Acetone and *tert*-butanol proved to be efficient solvents, and the acyl donor chain length did not significantly affect conversion yield. However, conversion yields were lower than those obtained in pyridine, in which the solubility of flavonoids was higher. In order to overcome the poor solubility of flavonoids in common organic solvents, Lambusta et al. (2003) used quercetin and catechin peracetates in alcoholysis reactions catalyzed by lipase B from *C. antarctica*, lipase from *Rhizomucor miehei* and lipase from *P. cepacia* [46].

Among the parameters affecting the conversion yield of enzymatic reactions, the water content is of great significance especially in the case of direct esterification. Kontogianni et al. showed that higher conversions were achieved in the dry solvents [45], and Gayot et al. (2003), in a deeper investigation, pointed out that dry 2-methyl-2-butanol and acyl acceptor (naringin), as well as water removal performed during the reaction with the use of molecular sieves significantly improved the conversion yield [47]. The nature of substrates is another parameter that significantly affects the performance of an enzymatic reaction. At a water-controlled environment, Ardhaoui et al. (2004) investigated the acylation of different flavonoids in 2-methyl-2-butanol with a broad spectrum of acyl donors [48]. Fatty acids with varying chain lengths (C6–C18) and degree of saturation, ω-substituted fatty and dicarboxylic acids, as well as phenolic acids and aryl-aliphatic acids were used and the effect of chain length, hydrophobicity, and substitution of acyl donor was pointed out [48–50]. Esters of phenolic acids were also used as acyl donors by Enaud et al. (2004) for the acylation of phloridzin [51]. These authors used solvent-free media and reduced pressure in order to eliminate by-product ethanol and shift the thermodynamic equilibrium toward ester synthesis, thus increasing conversion yields. On the same basis, reduced pressure was also applied for the acylation of naringin and rutin by fatty acid esters in 2-methyl-2-butanol [52]. In addition to the commonly employed flavonoids, several groups investigated the acylation of isolated substrates or fruit extracts [16,53–55] and also tested the biological activity of synthesized esters in terms of antioxidant activity toward lipoproteins [53], antimicrobial activity [53], and anti-tumor and -angiogenic activity [16]. The effect of substrate concentration [44,45,52,56], enzyme amount and reaction temperature [51] on conversion yield and regioselectivity were also investigated. More recently, De Oliveira et al. (2009) employed a combined docking, molecular mechanics, and molecular dynamics approach in order to explain the observed regioselectivity of *C. antarctica* lipase B at a molecular level [57].

This work underlines the biocatalytic preparation of novel bioactive analogs of selected flavonoids (silybin, rutin) and terpenoids (POH) in low-water media.

Enzymatic synthesis of flavonoid derivatives

The performance of an enzymatic reaction is affected by a plethora of factors (composition of the reaction media, nature of the reaction of acylation, operating conditions, nature and concentration of substrates, etc.). In order to investigate the effect of reaction medium on the performance of the enzymatic acylation of silybin with vinyl butyrate, several organic solvents were used. The (%) conversion of the

enzymatic acylation of silybin (30 mM) with vinyl butyrate (300 mM), which was catalyzed by Novozyme 435 at 50 °C after 96 h of incubation in *tert*-butyl alcohol, *tert*-amyl alcohol, acetonitrile, and acetone was 78.3, 94.5, 98, 100 %, respectively. It was found that the immobilized *C. antarctica* can catalyze the acylation of silybin in all of the reaction media tested with high conversion yields.

In all cases, a single product was detected by HPLC analysis, which indicates that this lipase-catalyzed reaction is regioselective. The higher reaction rate was observed in acetone (1.58 mmol h^{-1} g^{-1} of biocatalyst), while in the other solvents tested, the reaction rates were slightly lower. In all cases, studied higher conversion yields were obtained in transesterification reactions as compared to direct esterification (data not shown) [15]. The use of vinyl esters as acyl donors in biotransformations catalyzed by lipases is one of the most used methodologies for avoiding the release of free water into the reaction media [58,59] and ensuring a good irreversibility [60], due to the tautomerism of vinyl alcohol to acetaldehyde. In order to investigate the effect of molar ratio of acyl donor (vinyl butyrate) to silybin in acetone, the flavonolignan concentration was kept constant at 30 mM, while the amount of acyl donor gradually increased. The (%) conversion for the enzymatic transesterification of silybin with vinyl butyrate increased from 54 to 100 % when the molar ratio was increased from 1 to 10. Similar results were obtained using long-chain acyl donors, when acetone was used as reaction medium. The positive effect can be attributed to a thermodynamic shift of the equilibrium toward the synthesis of products due to acyl donor excess [61,62]. The increase of concentration from 10 to 80 mM led to a considerable increase of the amount of synthesized ester. It is noteworthy that when high substrate concentration (80 mM) was used, the amount of acylated derivative produced was significantly high (44.1 g l^{-1} after 96 h of incubation). However, as the solubility of the flavonoid is often low, the solubilization rate is the limiting step. The one-step regioselective acylation of rutin, with free mono- and polyunsaturated fatty acids, was also catalyzed by Novozym 435, verifying that *C. antarctica* exhibits selectivity toward not only primary OH groups, but toward secondary OH groups as well (Scheme. 2).

The effect of the acyl donor along with the alcohol donor used and the nature of the solvent on the flavonoid conversion were investigated. Several organic solvents were tested for the synthesis of rutin esters [16]. Acetone was chosen as the model solvent because it allows the satisfactory solubilization of both substrates. This solvent does not cause enzyme denaturation, and among the solvents tested in rutin esterification with oleic acid it was found to exhibit the higher reaction yield. Figure 1

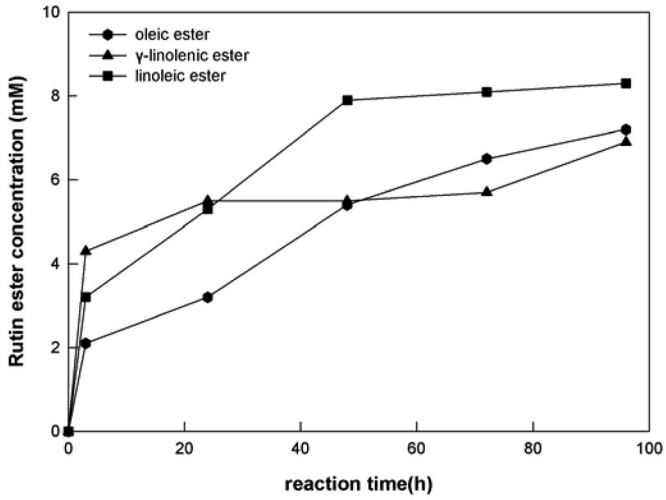


Fig. 1 Effect of the nature of the unsaturated fatty acids on the acylation of rutin with oleic acid, γ-linolenic acid, and linoleic acid. The reactions were carried out at 50 °C and 175 rpm by shaking a mixture of rutin (10 mM), unsaturated fatty acid (50 mM), and Novozyme 435 (11.1 mg ml^{-1}) in acetone (9.0 ml) for 96 h.

shows the influence of the nature of the unsaturated fatty acid. Our experiments indicated that the degree of the fatty acid saturation has only a slight effect on the conversion yield under operating conditions used, since the conversion yields of oleic acid, linoleic and γ-linolenic acid were found to be 70, 80, and 68 %, respectively.

Enzymatic synthesis of POH derivatives

The aim of the first modification of the monoterpene POH was to increase the lipophilic character, giving new physicochemical properties and consequently to improve the biological activity. The synthesis of POH esters by direct esterification was catalyzed by Novozyme 435.

For this purpose, fatty acids with chain length from 3 to 16 carbons were used. The reactions were carried out in mixtures of α-pinene and myrcene as well as in pure organic solvents. It was found that the esterification rates increased from 3.7 to 6.9 mM min^{-1} when using decreasing concentrations of myrcene in α-pinene (100–0 %) [17]. Higher rates and conversions were obtained when decanoic acid was used as the acyl donor (6.9 mM min^{-1}, 81.5 %, Fig. 2), while lower conversions were obtained with fatty acids of shorter (propionic acid, 5.6 %) or longer (hexadecanoic acid, 62.5 %) carbon chain. In addition, it was found that the effect of the water activity depended on the chain length of the acyl donor and the composition of the solvent mixture (data not shown) [17].

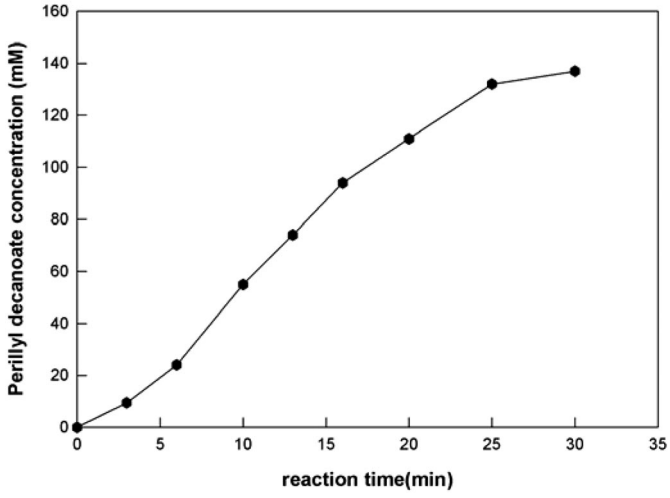
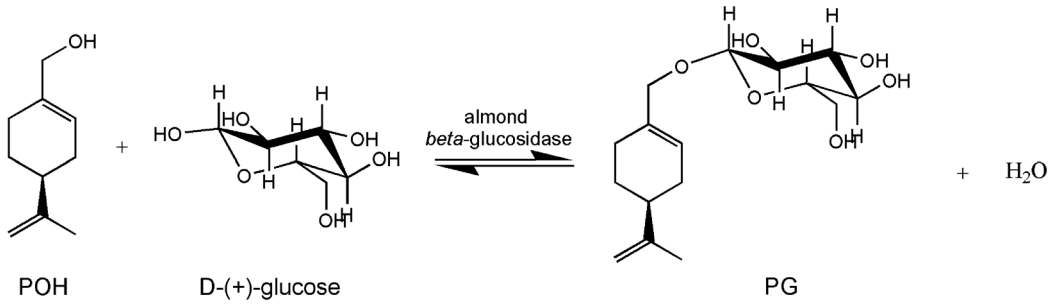


Fig. 2 Time course of POH acylation with decanoic acid. The reaction was carried out at 50 °C and 250 rpm by shaking a mixture of POH (167 mM), decanoic acid (167 mM), and Novozyme 435 (8 mg ml^{-1}) in α-pinene (5.0 ml) for 30 min.

Following the direct acylation of POH with a fatty acid, the enzymatic synthesis of two POH derivatives with a different polar and hydrophobic character, namely, PG and PGL, through a two-step modification was performed. Initially, glucosylation of POH on its active hydroxyl group with D-(+)-glucose was carried out (Scheme 4).



Scheme 4 Almond β-glucosidase-catalyzed synthesis of PG.

Among the various glucosidases tested, the best performance was observed for almond β-glucosidase. Complementary experiments were carried out in order to find out the optimum parameters of the procedure. The effect of pH of the aqueous phase, the influence of the temperature, the organic solvent, the substrate concentrations, and the organic-aqueous phase volume ratios were investigated [18]. Figure 3 shows the time course of the perillyl β-D-glucopyranoside concentration vs. time under the optimum conditions and the highest conversion of D-(+)-glucose obtained was 32 mM.

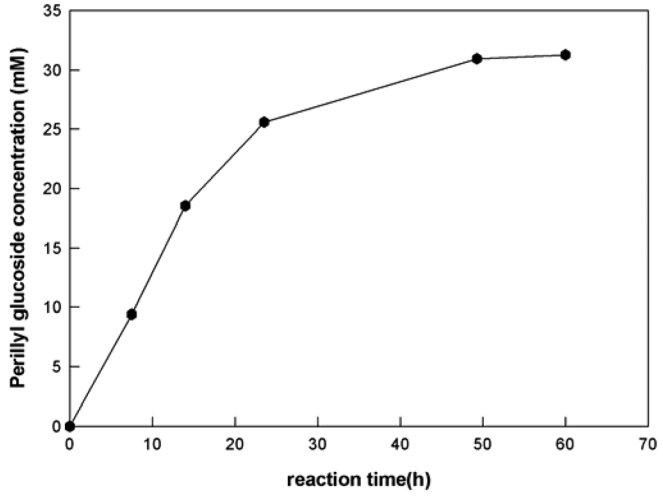
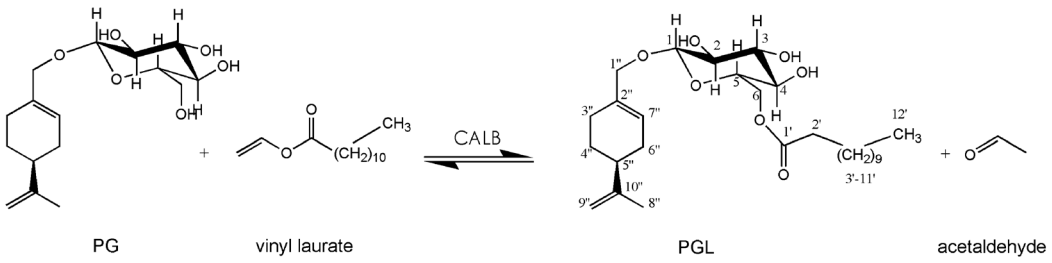


Fig. 3 Time course of POH glucosylation with D-(+)-glucose. The reaction was carried out at 50 °C and 200 rpm by shaking a mixture of D-(+)-glucose (0.4 M), POH (5.66 M), and almond-β-glucosidase (0.6 mg ml^{-1}) in a biphasic medium (5.0 ml, 90/10 organic-aqueous) for 60 h.

Following the glucosylation of POH with D-(+)-glucose, the transesterification of the glucoside with a medium-chain vinyl fatty ester was studied in order to produce an amphipathic derivative of PG (Scheme 5).



Scheme 5 Lipase-catalyzed synthesis of PGL.

Several commercially available lipases were screened for the synthesis of the desired PGL [18]. Novozyme 435 was found to have the higher affinity with the substrate reaching a 67.5 % conversion of the glucoside. Lipozyme (immobilized lipase from *R. miehei*) and lipase from *Candida rugosa* gave lower yields (44.7 and 18.9 %, respectively) while lipase from wheat germ, lipase from *Candida lipolytica*, lipase from *Rhizopus niveus*, lipase from *Mucor javanicus*, and lipase from *Rhizopus arrhizus* did not have any ability to catalyze the synthesis of PG laurate under this reaction approach. The effect of the acyl donor concentration on the performance of the biocatalytic process was also investigated. Under the optimum substrate concentration ratio 1/7 (perillyl glucoside/vinyl laurate) the final conversion of PG was 83.9 % (Fig. 4).

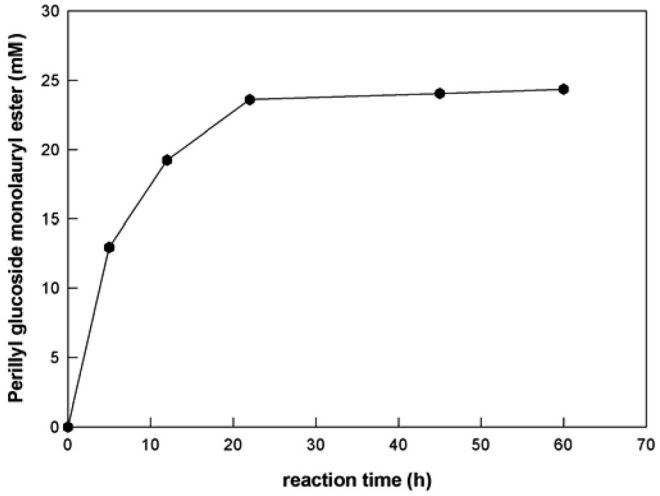


Fig. 4 The reaction was carried out at 50 °C and 1100 rpm by shaking a mixture of perillyl β-D-glucopyranoside (30 mM), vinyl laurate (100 mM) and Novozyme 435 (4 mg ml^{-1}) in dried *tert*-amyl alcohol (1 ml) for 60 h.

Biological actions of the novel derivatives

Effect of silybin derivatives on K562 cell proliferation

The anti-proliferative effect of silybin-23-*O*-butyrate and silybin-23-*O*-laurate, on K562 cells was investigated by using the MTT assay. Silybin, the parental molecule, was also included in the study as a positive control. As depicted in Fig. 5, all derivatives induced a concentration-dependent cell growth inhibition after 48 or 72 h, retaining in part the tumor growth inhibitory activity of the parental compound. The chain-length of the acyl donor had only a slight effect on the efficiency of the analogs.

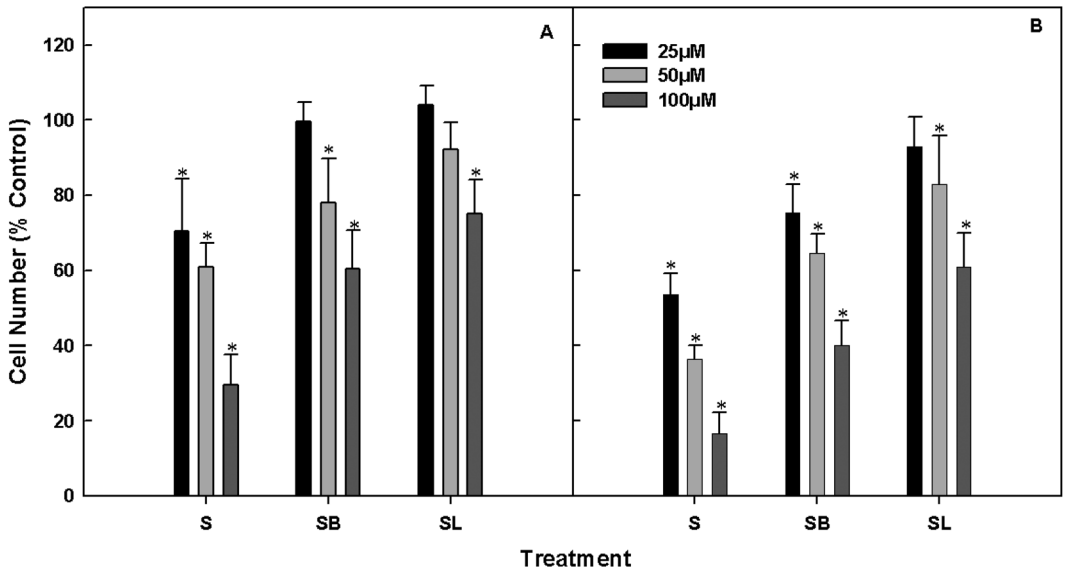


Fig. 5 Effect of silybin (S), silybin butyrate (SB), and silybin laurate (SL) on K562 cell growth. Cells (5×10^3/well) were plated in a 96-well plate, and 24 h later, they were treated with S, SB, SL, or 0.1 % DMSO vehicle (control). Cell numbers were measured after 48 h (A) or 72 h (B) by the MTT assay. Results are expressed as mean ±SEM of two independent experiments; $n = 12$; $^*p < 0.05$ from control.

Effect of rutin derivatives on VEGF release from K562 cells

VEGF is produced by most tumor cells and appears to be one of the main mediators of tumor-induced angiogenesis, an obligatory step for tumor growth and metastasis [63]. In these studies, in order to evaluate the potential anti-angiogenic activity of rutin derivatives, we examined their effect on the production of VEGF by K562 human leukemia cells (Fig. 6). As illustrated, incubation of cells with rutin es-

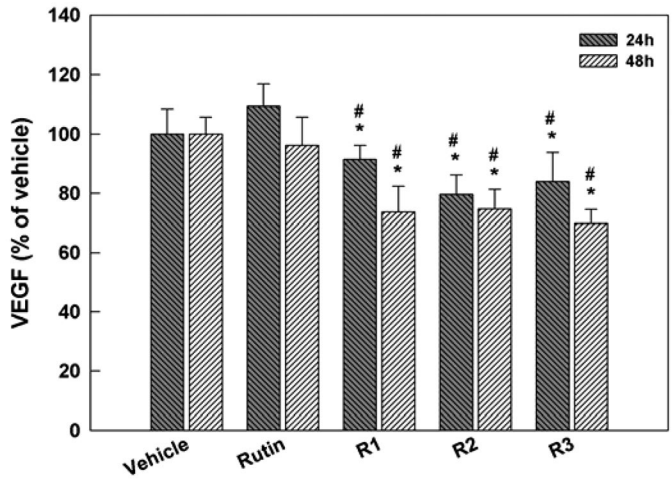


Fig. 6 Effect of flavonoid esters on VEGF release from K562 cells. K562 cells (10^6) were treated with rutin, rutin esters with oleic acid (R1), linoleic acid (R2), γ-linolenic acid (R3) or 0.1 % v/v DMSO vehicle. At the indicated times, culture supernatants were collected and measured by ELISA for the presence of VEGF. Results are expressed as mean ±SEM; $n > 9$, $p < 0.05$ from control (*) and rutin (#).

ters induced a significant decrease in the secretion of VEGF, while the parental molecules (rutin and unsaturated fatty acids) alone were not effective. Consistent with these results, rutin has been previously shown to be inefficient on an ex vivo angiogenesis assay [64], whereas the effect of polyunsaturated fatty acids, including linoleic acid, on tumor cell growth has been reported to be either inhibitory or stimulatory depending on cancer cell type [65,66]. Therefore, the analogs derived from rutin enzymatic esterification presented new anti-angiogenic properties and thus may have potential application in tumor prevention [16].

Effect of perillyl derivatives on cell proliferation

As mentioned previously, the enzymatic esterification of POH on the unique primary hydroxyl group of the molecule leads to the abolishment of its anti-tumor and -angiogenic activities [67]. The biological effect of POH glucoside and POH glucoside monolauryl ester, derived from the glucosylation of the hydroxyl moiety and the subsequent acylation of the derived glucoside, was further analyzed on the proliferation of mouse LLC cells. It was found that, in contrast to the direct acylation of POH, the glucosylated analog retains a part of its biological activity. Furthermore, the subsequent acylation of PG resulted in the improvement of its anti-proliferative activity at levels comparable to that of the POH (Fig. 7) [18]. These findings provide new insights into structure/biological function relationships and are important for future design of novel derivatives with improved and/or modified activities.

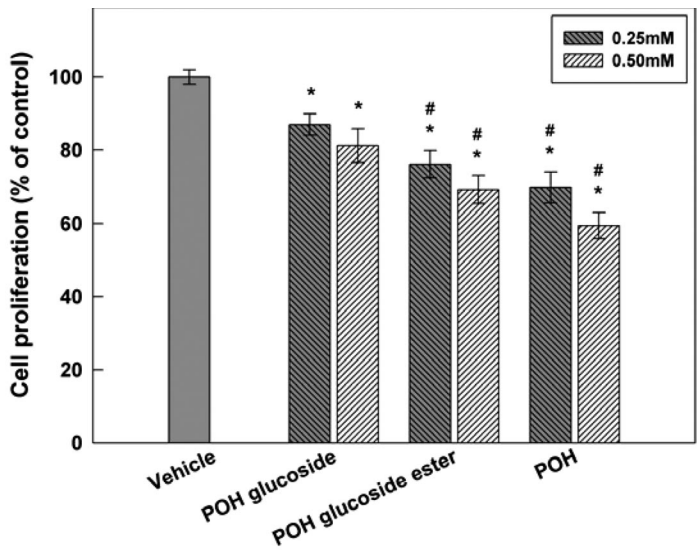


Fig. 7 Effect of POH glucoside and POH glucoside ester on LLC cell growth. Cells (3×10^3/well) were plated in a 96-well plate and after 24 h, were treated with POH, POH analogs, or vehicle (control) for 24–48 h. Cell numbers were determined by the CyQUANT cell proliferation assay. Results are expressed as mean % of control ±SEM of two independent experiments; $n = 12$; $^*p < 0.05$ from control; $\#p < 0.05$ from POH glucoside.

CONCLUSIONS

The primary intent of this review article has been to demonstrate that biocatalysis can play a decisive role in the drug discovery process. Although the replacement of well-established chemical processes for the synthesis of novel bioactive compounds with biocatalytic ones is advantageous from a societal and environmental viewpoint, enzyme performance must still improve in order to fulfill industrial needs. Medium engineering strategies and the advent of modern biotechnology, particularly recombinant DNA technology, has not only made the enzymes economically feasible but optimized their performance

under industrial conditions. Our results indicate that novel derivatives of flavonoids and terpenoids can be efficiently synthesized using hydrolases by introducing selective modifications that can alter their physicochemical and biological properties. It is noteworthy that the new derivatives described herein exhibit new in vitro anti-carcinogenic effects and can constitute a valuable tool for elucidating the pathways involved in the overall carcinogenesis process. Since results from in vitro experiments cannot be directly extrapolated to clinical effects, more studies are required to clarify the mechanisms of action and evaluate their potential as anticancer agents.

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