Potential *O*-Acyl-Substituted (−)-Epicatechin Gallate Prodrugs as Inhibitors of DMBA/TPA-Induced Squamous Cell Carcinoma of Skin in Swiss Albino Mice

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(−)-Epicatechin-3-gallate (**1**) is one of the principal catechins of green tea and exhibits cancer-preventive activities in various animal models. However, this compound is unstable in neutral or alkaline medium and, therefore, has a poor bioavailability. To improve its stability, *O*-acyl derivatives of **1** were prepared by isolating the partially purified tea catechin fraction from green tea extract and treating it with a variety of acylating agents. The resulting derivatives, compounds **2**–**6**, were screened for their antitumor potential against 7,12-dimethylbenz[*a*]anthracene (DMBA)/12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced squamous cell carcinogenesis of skin in mice. The results showed that the antitumor activity decreased with the increase in size of the chain length of the acyl groups, *i.e.*, from compound **2**, derivative with an Ac group, to compound **6**, possessing a valeryl group. Moreover, the C_4 derivative with a branched acyl chain, **5**, had a lower activity than the linear C_4 derivative **4**. This reduction in the inhibitory activity may be due to the steric hindrance by the two Me groups. Moreover, significant increases in the protein levels analyzed by ELISA of c-Jun, p65, and p53 were observed in the skin of DMBA/TPA treated mice, whereas mice treated with **2** and DMBA/TPA had a similar expression of these transcription factors than the control mice. The prodrug potential of the *O*-acyl derivatives **2**–**6** showed that they were adequately stable to be absorbed intact from the intestine, more stable at gastric pH, and suitable for oral administration.

Introduction. – Green tea derived from the leaves of *Camellia sinensis* has been under extensive investigations for its health benefits, and there is evidence that various catechins (polyphenolic compounds) are responsible for its biological activities. (−)-Epicatechin-3-gallate ((−)-ECG, **1**; *Fig. 1*), one of the principal catechins of green tea, has shown cancer preventive activities in various animal models [1–5]. Compound **1** is hydrophilic and unstable in neutral and alkaline media and its bioavailability is poor [6]. Glucuronidation, methylation, sulfation, and microbial degradation in the colon are major barriers for its bioavailability [7][8]. These factors limit further development of catechins as therapeutic agents. To overcome the aforementioned drawbacks, the prodrug concept can be used, which involves the development of chemically modified derivatives of the pharmacologically active agent that must undergo transformation *in vivo* to release the active drug. Such chemical alterations of the physicochemical properties impart the stability and consequently improve the bioavailability and thereby increase the developability and usefulness of a potential molecule. In most cases, prodrugs are simple chemical derivatives that require only one to two chemical or

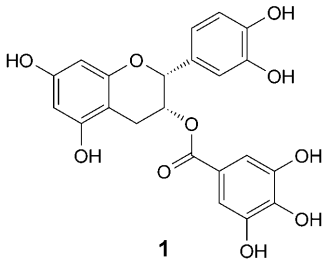


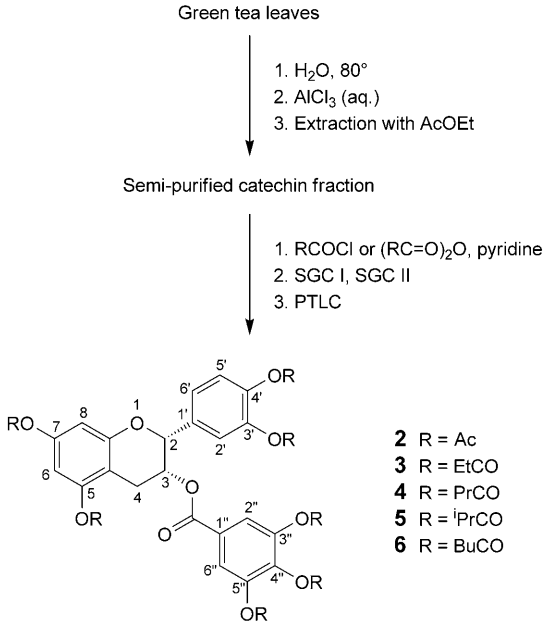
Fig. 1. *Chemical structure of (−)-epicatechin-3-gallate* (**1**)

enzymatic (*e.g.*, by esterases and phosphatases) transformation steps to yield the active parent drug. In some cases, a prodrug may consist of two pharmacologically active drugs that are coupled together in a single molecule so that each drug acts as a promoiety for the other. Currently, 5–7% of the drugs approved worldwide can be classified as prodrugs, and *ca.* 15% of all new drugs approved in 2001 and 2002 were prodrugs [9]. Esters are the most common prodrugs used, and it is estimated that *ca.* 49% of all marketed prodrugs are activated by enzymatic hydrolysis. Ester prodrugs are accomplished by masking of the polar groups, resulting in enhanced lipophilicity and making OH groups unavailable for phase II biotransformation or oxidative degradation [10].

A number of studies have been carried out for the preparation and evaluation of acylated derivatives as potential catechin prodrugs with improved stability, bioavailability, and pharmacological activities [11–26]. Most of the investigators have carried out their studies with commercially available tea catechins as starting material for the synthesis of *O*-acyl derivatives. Previously, we have reported the semisynthetic synthesis and antitumor evaluation of *O*-acyl derivatives of (−)-epigallocatechin-3-gallate [15]. In the present study, we report the semisynthetic preparation of *O*-acyl derivatives of (−)-epicatechin-3-gallate ((−)-ECG), **2**–**6**, from the partially purified catechin fraction and their inhibitory effect on 7,12-dimethylbenz[*a*]anthracene (DMBA)/12-*O*-tetradecanoylphorbol-13-acetate (TPA) induced squamous cell carcinoma of skin in Swiss albino mice. The effect of the most active derivative, *i.e.*, the *O*-acetyl derivative **2**, on the expression of various transcriptional factors (AP-1, NF-κB, and p53) was also investigated, and the physicochemical properties of compounds **2**–**6** were evaluated to assess their prodrug potential.

Result and Discussion. – *Chemistry.* Five potential *O*-acyl-substituted (−)-ECG prodrugs, **2**–**6**, were synthesized by treatment of the partially purified catechin fraction of green tea leaves with various acylating agents, *i.e.*, Ac_2O, propionic anhydride, butyric anhydride, isobutyric anhydride, and valeroyl chloride (*Scheme*). The dry green tea leaves were extracted with hot H_2O. The extract was treated with an aqueous $AlCl_3$ solution to precipitate the tea catechins as Al–catechin complexes. The complexes were decomposed with H_2SO_4, and the liberated catechins were extracted with AcOEt. The removal of the solvent under reduced pressure gave a partially purified catechin fraction as brown colored semisolid material, which was washed with small amounts of $CHCl_3$ to remove any traces of caffeine.

Scheme. *Semisynthetic Synthesis of the O-Acyl Derivatives of (−)-Epicatechin-3-gallate,* **2**–**6**



For the synthesis of the *O*-acyl derivatives, the partially purified catechin fraction was treated with an acylating agent in the presence of pyridine (*Scheme*). The acylated material was subjected to tandem preparative-scale elution chromatography (SGC I and SGC II) [27] and preparative TLC (PTLC), to obtain the *O*-acyl derivatives of (−)-ECG present in green tea. The structures of these compounds were determined by physical data and spectral studies. The relative R_f values show that the polarity of the *O*-acyl derivatives decreased with the increase in size of the chain length of the acyl groups, *i.e.*, from compound **2**, derivative with an Ac group, to compound **6**, possessing a valeroyl group. The *O*-acyl derivatives of (−)-ECG were obtained as semisolids, except the *O*-isobutyryl derivative **5**, which was obtained as white flakes with a m.p. of 178–180°. The optical rotations ($[\alpha]_D^{25}$) of the derivatives were −27.0, −31.81, −20.0, −27.8, and −30.7 ($CHCl_3$), respectively. The UV spectra showed the characteristic bands of (acyloxy)phenyl rings (260–270 nm) and a band in the 216–228-nm region for the substituted gallate moiety. The IR spectra showed bands at *ca.* 1673–1428 cm^{-1}, indicative for the presence of aromatic rings, whereas the bands in the range of 1764–1775 cm^{-1} were characteristic for the presence of acyl functional groups. The bands in the range of 1723–1727 cm^{-1} were characteristic for the BzO group. In the ^{1}H-NMR spectra, for all *O*-acyl derivatives of (−)-ECG, **2**–**6**, a broad *singlet* appeared in the range of δ(H) 4.97–5.19 for H–C(2) and a *multiplet* in the range of δ(H) 5.37–5.62 for H–C(3). Other signals appeared in the range of δ(H) 2.91–3.07 (*dd*, J = 4.2–4.6, 17.6–18.1) for H_a–C(4), 2.81–2.98 (*dd*, J = 1.5–2.5, 17.5–18.0) for H_b–C(4), 6.65–6.73 (*d*, J = 2.2) for H–C(6), and 6.49–6.59 (*d*, J = 2.2) for H–C(8). The signals for the ring *B*

H-atoms appeared at δ(H) 7.30–7.33 (*d*, J = 2.0–2.1) for H–C(2′), 7.15–7.20 (*d*, J = 7.45–7.62) for H–C(5′), and 7.26–7.28 (*d*, J = 1.94–2.00) for H–C(6′). The signals for the ring *D* H-atoms appeared at δ(H) 7.60–7.61 as *singlet* for H–C(2″) and H–C(6″). The signals of the alkyl H-atoms of the acyl chains appeared in the range of δ(H) 2.22–2.28 as overlapping *singlets* for the seven MeCO of the *O*-acetyl derivative of (−)-ECG, **2**, 1.21–1.28 for the seven *Me*CH_2CO and 2.49–2.62 for the seven MeCH_2CO of *O*-propionoyl derivative **3**, 0.73–1.44 for the seven *Me*CH_2CH_2CO, 1.7–2.1 for the seven MeCH_2CH_2CO, and 2.4–2.5 for the seven $MeCH_2CH_2CO$ of *O*-butyryl derivative **4**, 1.25–1.34 for the seven Me_2CHCO and 2.68–2.83 for the seven Me_2CHCO of *O*-isobutyryl derivative **5**, and 0.91–0.95 for the seven *Me*$CH_2CH_2CH_2CO$, 1.35–1.39 for the seven Me$CH_2CH_2CH_2CO$, 1.63–1.71 for the seven $MeCH_2CH_2CH_2CO$, and 2.41–2.50 for the seven $MeCH_2CH_2CH_2CO$ of *O*-valeroyl derivative **6**. In the ^{13}C-NMR spectra, the signals of the CO C-atoms of the acyl groups appeared in the range of δ(C) 176.5–163.1 and the signals for the C-atoms adjacent to the CO group (O=C–*C*) at δ(C) 21.0–20.1, 29.6–27.1, 36.2–35.5, 34.2–33.8, and 33.7–33.2 for compounds **2**–**6**, respectively. The mass spectra showed the molecular-ion peaks ($[M+NH_4]^+$) at *m*/*z* 754.16, 852.71, 950.38, 950.38, and 1048.49 for compounds **2**–**6**, respectively. The purity of the compounds was verified by HPLC (*Fig. 2*).

Antitumor Activity of the O-*Acyl Derivatives of (−)-Epicatechin-3-gallate,* **2**–**6**. Cancer is a multistep process involving the sequential phases of initiation, promotion, and progression. The mouse skin carcinogenesis model is a well-characterized model for studying the genetic and biological changes associated with the chemical initiation of the lesions and their subsequent transition to squamous cell carcinoma. The various *O*-acyl derivatives of (−)-ECG, **2**–**6**, were screened for their tumor inhibitory potential against 7,12-dimethylbenz[*a*]anthracene (DMBA)/12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced skin carcinogenesis in Swiss albino mice. The administration of **2**–**6** at the maximal tolerated dose of 50 mg/kg body weight showed a significant delay in the onset and an overall reduction of papillomas as well as a better survival rate in comparison to the treatment with DMBA/TPA alone, suggesting an antitumor activity of the (−)-ECG derivatives. The onset of papillomas was observed in the fifth week in the DMBA/TPA treated mice and found to be 18%. There was a gradual rise in the incidence of tumors that reached 100% during the 14th week (*Fig. 3*). The incidence of DMBA/TPA-induced papillomas was delayed for 4 weeks by **2**–**4** and by 2 weeks for **5** and **6**. Moreover, as shown in *Fig. 3*, compounds **2**–**6** showed a significant decrease in the incidence of cancer (34, 39, 44, 48, and 58% *vs.* 100%, resp.) at the end of 19 weeks, as compared with DMBA/TPA alone ($P<0.001$, $P<0.01$). The antitumor profile of these compounds showed that there was a significant decrease in the activity with the increase in size of the chain length of the acyl groups ($P<0.01$). Compound **2** showed a better antitumor activity than all other derivatives ($P<0.001$), and compound **6** was the least active compound among them.

The survival rate of the mice decreased significantly in the DMBA/TPA-treated mice, as compared with the vehicle-treated group (30 *vs.* 80%). The survival rate of the animals treated with the (−)-ECG derivatives was significantly higher in comparison to the DMBA/TPA-treated group (*Fig. 4*). The group treated with the acetyl derivative **2** showed a survival rate of 70%, whereas those treated with **3**–**6** showed a survival rate of 60% ($P<0.05$).

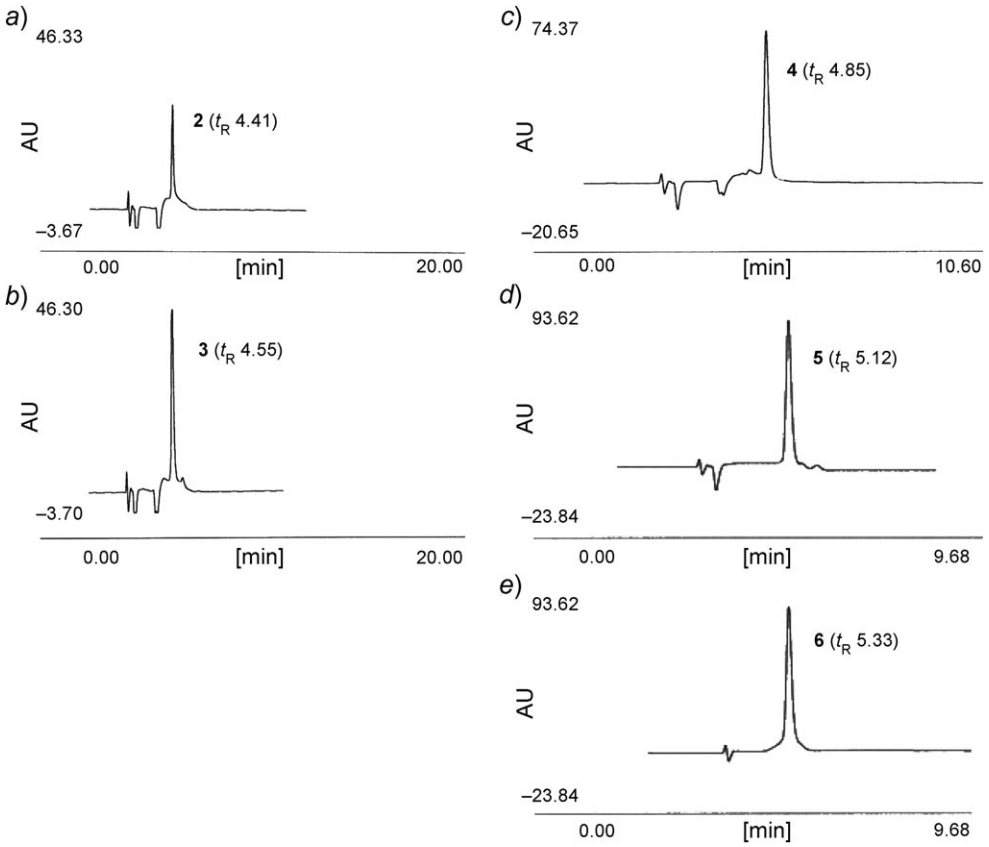


Fig. 2. *HPLC Chromatograms of the* a) O-*acetyl,* b) O-*propionyl,* c) O-*butyryl,* d) O-*isobutyryl, and* e) O-*valeroyl derivatives of (−)-epicatechin-3-gallate*

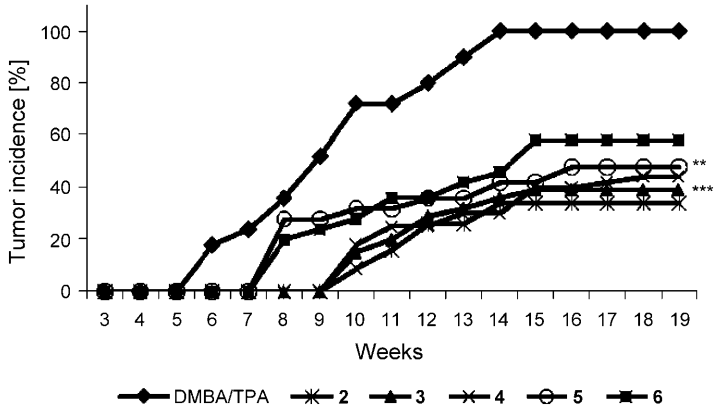


Fig. 3. *Effect of the* O-*acyl derivatives of (−)-epicatechin-3-gallate,* **2**–**6**, *on the incidence of DMBA/TPA-induced tumors in mice.* Significant differences compared to DMBA/TPA treatment alone: $P < 0.01$ (**), $P < 0.001$ (***).

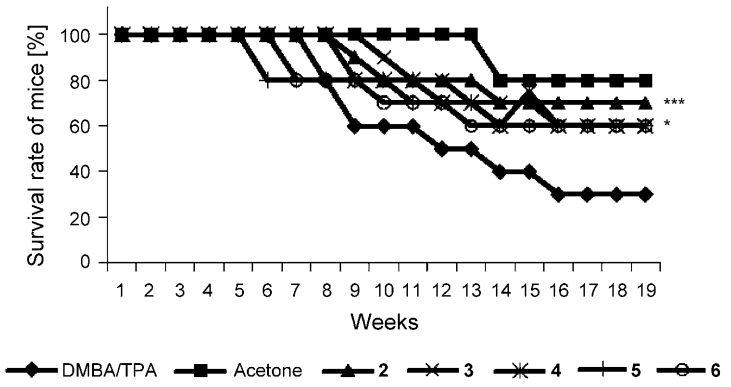


Fig. 4. *Effect of the* O*-acyl derivatives of (−)-epicatechin-3-gallate,* **2**–**6***, on the survival rate of DMBA/TPA treated mice.* Significant differences compared to DMBA/TPA treatment alone: $P<0.05$ (*), $P<0.001$ (***).

The average body weight of the mice treated with **3**, **4**, **6**, or DMBA/TPA alone did not differ from that of the acetone-treated mice throughout the study. However, there was a slight increase in the average body weight of the mice treated with **2** and a slight decrease in the average body weight of the **5**-treated mice at the end of the study ($P<0.001$ and $P<0.05$, resp.). The histopathological examination of the depilated backs of the mice revealed normal skin and subcutaneous tissue in the acetone-treated mice, whereas the DMBA/TPA-treated animals showed well-differentiated squamous cell carcinoma with formation of keratin pearls. There was a marked infiltration of cancer cells in the underlying dermis. The skin section of the (−)-ECG *O*-acyl derivative-treated mice showed hyperplastic papillomatous lesions without evidence of infiltration or cytological atypia.

The results showed that the antitumor activity decreased with the increase of the chain length of the acyl groups, *i.e.*, from compound **2**, substituted with an Ac group, to compound **6**, bearing a valeryl group. Moreover, the compound with a branched C_4 acyl chain (**5**) had a slightly lower inhibitory activity than compound **4**, with a linear C_4 acyl chain. This may be due to the steric hindrance by the two Me groups. Indeed, this might inhibit the enzyme-catalyzed hydrolytic cleavage of the ester bond of the *O*-isobutyryl derivative of (−)-ECG (**5**). The pretreatment of the animals with the (−)-ECG derivatives showed their ability to interfere with the initiation of the tumors, which is a relatively rapid process, and the continuous treatment with the (−)-ECG derivatives after the application of TPA interferes with the tumor promotion, which is a slow process [28]. The compounds also increased the survival rate of the animals. This effect could be associated with the lower papilloma burden as a result of the inhibitory effect of the compounds on the carcinogenesis. The slight weight gain with compound **2** could be the result of recovery from the effect of DMBA/TPA or, alternatively, the better papilloma control.

Effect of Compound **2** *on the Expression of Transcription Factors.* Several transcription factors, including the activator protein-1 (AP-1), the nuclear factor kappa B (NF-κB), and the signal transducers and activators of transcription (STATs), have been linked to tumor promotion [29][30]. AP-1 is an important nuclear

transcription factor involved in many cellular functions, such as cell proliferation, death, survival, and differentiation [31]. The transcription factor AP-1 consists of homodimers and heterodimers composed of basic region leucine zipper protein that belongs to the Jun (c-Jun, JunB, and JunD), Fos (c-Fos, Fos B, Fra-1, and Fra-2), Jun dimerization partners (JDPs and JDP2), and the closely related activation transcription factors. AP-1 Proteins have target genes regulating cell proliferation and death. The Jun proteins control cell life and death through their ability to regulate the expression and function of cell cycle modulators. A functional role for AP-1 components in the epidermis of the skin has been suggested for differentiation, carcinogenesis, UV-response, photo-aging, and wound repair [30]. This might be due to the regulation of AP-1, which can be achieved at different levels, by changes in the transcription of genes encoding AP-1 subunits, by controlling the stability of the mRNAs, by posttranslational processing, turnover, and modification by phosphorylation of AP-1 proteins, and by specific interaction between AP-1 proteins and other transcription factors and cofactors [32]. There was a significant increase ($P<0.05$) in the level of c-Jun protein as analyzed by ELISA in the skin of DMBA/TPA treated mice (*Group II*) as compared to the control mice skin of *Group I* (*Table 1*). Moreover, there was a significant decrease ($P<0.001$) observed in the level of c-Jun in the mice treated with compound **2** and DMBA/TPA as compared to the DMBA/TPA treated mice.

Table 1. *Effect of Compound* **2** *on the Expression of Transcription Factors in the Skin of DMBA/TPA Treated Mice Analyzed by ELISA*

Transcription factor	Protein expression [μg/ml][a])		
	Control	DMBA\TPA	**2** + DMBA\TPA
AP-1 (c-Jun)	0.223 ± 0.08	0.562 ± 0.01*[b])	0.208 ± 0.01***[c])
NFκB (p65)	0.156 ± 0.02	0.365 ± 0.07**[b])	0.116 ± 0.04*[c])
P53	0.228 ± 0.05	0.742 ± 0.04*[b])	0.308 ± 0.02*[c])

[a]) Values are means ± standard deviations of six independent observations. [b]) Significant differences compared to control group (*Group I*): *, $P<0.05$; **, $P<0.01$. [c]) Significant differences compared to DMBA/TPA treated mice (*Group II*): *, $P<0.05$; ***, $P<0.001$.

NF-κB has been implicated in carcinogenesis, as it plays a critical role in cell survival, cell adhesion, inflammation, differentiation, and cell growth. Cancer is a hyperproliferative disorder that results from tumor initiation and promotion and ultimately produces tumor metastasis. Several genes involved in cellular transformation, proliferation, invasion, and angiogenesis are regulated by NF-κB [33]. Oxidant stress can result in the degradation of cytoplasmic NF-κB inhibitor (IκB) and its translocation to the nucleus [34]. In the present study, the protein expression of p65, a subunit of the NF-κB complex, increased significantly in the DMBA/TPA treated mice (*Table 1*). This indicates an activation of NF-κB, which was probably due to the inhibition of IκB protein that resides in the cytoplasm and, hence, increased the level of p65 expression in the nuclear fraction. The constitutive activation of NF-κB also appears to play a role in cell proliferation [35]. A significant decrease ($P<0.05$) was observed in the level of p65 in the skin nuclear fraction of the mice treated with **2** and

DMBA/TPA as compared to that of DMBA/TPA treated mice (*Table 1*). This may be due to the suppression of NF-κB by this compound. Several chemopreventive agents are inhibitors of NF-κB activation. These inhibitors can block anyone or several steps in the NF-κB signaling cascade, the translocation of NF-κB into the nucleus, DNA binding of the dimers, and/or interactions with the basal transcription activation.

The tumor suppressor gene p53 is regarded as a key factor in maintaining the balance between cell growth and cell death [36][37]. Due to its role in the regulation of the cell cycle, alterations in p53 are critical events in carcinogenesis [38]. In the present study, a significant increase in the p53 expression was observed in the skin nuclear fraction of DMBA/TPA treated mice (*Group II*) compared to the control group (*Group I*). Moreover, there was a significant decrease ($P<0.05$) observed in the level of p53 in the skin nuclear fraction of mice treated with **2** and DMBA/TPA as compared to that of the DMBA/TPA treated mice (*Table 1*).

Physicochemical Properties and Enzymatic Hydrolysis. In addition to an optimum solubility and lipophilicity required for a good bioavailability, the prodrug derivatives should be sufficiently chemically stable and bio-labile to get converted into the parent drug molecules once absorbed. Therefore, to assess the prodrug potential of the *O*-acyl derivatives of (−)-ECG, **2**–**6**, studies on their physicochemical properties, including solubility, partition coefficient, and chemical stability, and enzymatic hydrolysis were carried out.

The gastrointestinal absorption of most drugs depends on the passive transport and therefore on the combined effects of aqueous solubility and partition coefficients. On the basis of the available data, drugs having logarithmic octanol/H_2O partition coefficients (log *P* values)$\geq$2 and aqueous solubilities higher than 10 μg/ml are assumed to be well absorbed [39–42]. Needless to say that kinetic factors, such as transport and dissolution rates, are very important in addition to the abovementioned thermodynamic factors for estimating the absorption. The poor bioavailability of (−)-ECG is due to the low lipophilicity and instability, which have limited its development for clinical use. Esterification of the OH groups gave esters having fair solubilities in buffer (pH 7.4) and a greater lipophilicity. The solubilities of the *O*-acyl derivatives of (−)-ECG, **2**–**6**, were found to be between 1.05 and 32.23 μg/ml and the log *P* values between 2.42 and 6.08 (*Table 2*). The solubility of the *O*-acyl-(−)-ECG derivatives **2**–**6** decreased from 32.23 to 1.05 μg/ml with the increase in size of the chain length of the acyl groups. Similarly, the lipophilicity (log *P*) of derivatives **2**–**6** increased from 2.42 to 6.08 as the side chain length increased. The solubility decreased while the lipophilicity increased due to the chain length of the acyl groups. These results indicated that the potential prodrugs **2**–**6** might be suitable for oral administration.

Table 2. *Lipophilicity* (log *P*) *and Solubility of the* O-*Acyl Derivatives of (−)-Epicatechin-3-gallate*

Compound	log *P*	Solubility [μg/ml]
2	2.42 ± 0.02	32.23 ± 0.44
3	4.84 ± 0.05	22.61 ± 0.43
4	5.08 ± 0.03	13.86 ± 0.36
5	5.40 ± 0.05	2.88 ± 0.34
6	6.08 ± 0.05	1.05 ± 0.38

Prodrug derivatives should be sufficiently stable, so that they can be formulated in a stable dosage form [42]. For a better absorption from the stomach, the drug should be stable at gastric pH (pH 1–2). To assess these requirements, hydrolysis studies were carried out in both phosphate buffer at pH 7.4 and HCl buffer at pH 2.0 [43]. The phosphate buffer at pH 7.4 represented the pH in the intestine. The hydrolysis rate was monitored by the appearance of the parent compound. The prodrugs followed pseudo-first order rate kinetics for several half-lives, and the rate constants (k) and half-lives ($t_{1/2}$) for the prodrugs in both phosphate and HCl buffer are shown in *Table 3*. The rates of hydrolysis at pH 7.4 decreased from 0.0199 to 0.0148 h^{-1}, whereas the rates of hydrolysis at pH 2.0 increased from 0.0048 to 0.0060 h^{-1}, as the side chain length of the *O*-acyl-(−)-ECG derivatives **2**–**6** increased. Hence, the $t_{1/2}$ of the *O*-acyl derivatives at pH 7.4 and pH 2.0 increased and decreased, respectively, as the acyl chain length increased from an Ac to a valeroyl group, as shown in *Table 3.* These results indicated that derivatives **2**–**6** might be adequately stable to be absorbed from the intestine and that they are more stable at gastric pH, from which they will be absorbed in unionized form.

Table 3. *Hydrolysis Rates and Half-Lives at pH 7.4 and 2.0 of the* O-*Acyl Derivatives of (−)-Epicatechin-3-gallate*

Compound	$k_{pH\ 7.4}$ [h^{-1}]	$k_{pH\ 2.0}$ [h^{-1}]	$t_{1/2\ (pH\ 7.4)}$ [h]	$t_{1/2\ (pH\ 2.0)}$ [h]
2	0.0199	0.0048	34.8	144.4
3	0.0182	0.0050	38.1	138.6
4	0.0176	0.0051	39.8	135.9
5	0.0162	0.0047	42.8	147.5
6	0.0148	0.0060	46.8	115.5

The enzymatic cleavage of the *O*-acyl derivatives of (−)-ECG, **2**–**6**, was assessed in 80% human plasma. It was observed that these derivatives were readily hydrolyzed (*Table 4*). The hydrolysis followed pseudo-first order rate kinetics for several half-lives. An essential prerequisite for the successful use of a prodrug is its ability to readily release the parent drug after oral administration [41][42]. The rates of hydrolysis of the *O*-acyl-(−)-ECG derivatives in 80% human plasma were found to be 2.8–2.5 h^{-1} and the $t_{1/2}$ 15.4–16.3 min. The slightly higher hydrolysis half-life of the *O*-isobutyryl derivative **5** compared to the *O*-butyryl and *O*-valeroyl derivatives, **4** and **6**, respectively, may be due to steric hindrance of the Me groups in the isobutyryl derivative. These observations indicated that compounds **2**–**6** are readily hydrolyzed in

Table 4. *Hydrolysis Rate and Half-live of the* O-*Acyl Derivatives of (−)-Epicatechin-3-gallate in 80% Human Plasma*

Compound	k_{plasma} [h^{-1}]	$t_{1/2\ (plasma)}$ [min]
2	2.8	14.6
3	2.7	15.4
4	2.6	15.9
5	2.4	16.7
6	2.5	16.3

plasma to release the parent drug molecule. The rapid rate of hydrolysis observed in 80% human plasma compared to the slow rate of hydrolysis in absence of plasma under similar conditions in buffer solution (pH 7.4) was important for these compounds. This implies that the enzymatic reactivity of **2**–**6** is independent of their intrinsic ester reactivity.

Conclusions. – There is enough evidence that (−)-ECG is a potent catechin present in green tea and that it is responsible for the antitumor properties of its potential prodrugs **2**–**6** after their transformation to release the parent molecule. However, (−)-ECG has a poor bioavailability, due to its instability and poor aqueous solubility, and such compounds are difficult to formulate in therapeutic dosage forms for clinical use. Many investigators have prepared (−)-ECG derivatives to overcome these problems using commercially available (−)-ECG as the starting material. We have devised a method for the derivatization of (−)-ECG from the partially purified catechin fraction of green tea by the use of acyl chlorides/anhydrides to impart the stability, followed by isolation of these derivatives. The results of antitumor activity showed that the dose required in an *in vivo* cancer model is quite high (50 mg/kg) and further modification and optimization of the pharmacophore of (−)-ECG derivatives is required to develop them as potent clinical antitumor agents.

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Experimental Part

General. The acylating agents (Ac_2O, propionic anhydride, butyric anhydride, isobutyric anhydride, and valeroyl chloride) and the solvents (AR or HPLC grade) were purchased from *E. Merck*/*S.D. Fine Chemicals Ltd.*, India. Column chromatography (CC): silica gel (SiO_2; 60–120 mesh; *E. Merck*, India). TLC: precoated *G* silica-gel plates of appropriate sizes (0.2 mm thickness, plastic base; *E. Merck*, India). HPLC: *LiChrosphere* C_{18} column (250 × 4.6 mm; *E. Merck*, India), *Waters* HPLC system equipped with an automated gradient controller, a *510* pump, a *U6K* injector, a *481* UV detector, and a 746 data module (*Waters*, USA). M.p.: *Boetius* stage apparatus; uncorrected [33]. Optical rotations: *Rudolph Autopol III* automatic polarimeter (*Rudolph*, USA). UV Spectra: *Perkin Elmer LAMBDA-15* spectrophotometer (*Bodenseewerke*, *Perkin-Elmer & Co. GmbH*, Germany). IR Spectra: *Perkin Elmer RX-1* spectrometer; $\tilde{\nu}$ in cm^{-1}. ^1H- and ^{13}C-NMR spectra: *Bruker AC 30*-NMR spectrometer, at 400 and 75 MHz, resp., in $CDCl_3$; δ in ppm rel. to Me_4Si, *J* in Hz. LC/ESI-MS: *LC Waters Allianz 2695*; mobile phase, ammonium acetate buffer pH 6.7/MeCN 1:9; software, MassLynx 4.0 (*Waters*, USA). The spectra were recorded in positive ion mode between *m*/*z* 120 and 1500.

Plant Material. Green tea was collected at the *Himachal Pradesh Krishi Vishvavidyalya* (HPKV), accredited institution of the Indian Council of Agriculture Research, Palampur, Himachal Pradesh, India. A voucher specimen (No. 1453) was deposited with the Herbarium of the University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh, India.

Extraction and Isolation of the Partially Purified Catechin Fraction. Dry green tea leaves (20 g) were grounded to coarse powder, transferred to a conical flask, and soaked in H_2O (500 ml) at r.t. for 1 h. The contents were heated at 80° for 1 h. The mixture was cooled to r.t. and filtered. The filtrate was treated with 15 ml of an aq. $AlCl_3$ soln. (20% (*w*/*v*)) and 15 ml of a sat. $NaHCO_3$ soln. to adjust the pH to 5.5–6.5. The mixture was allowed to stand for 1 h (25°). The precipitated material was collected by filtration and then suspended in H_2O (20 ml) and H_2SO_4 (5 ml, 40% (*w*/*v*)). The mixture was extracted with AcOEt (3 × 100 ml), and the org. layer was dried (anh. Na_2SO_4). The solvent was removed under reduced pressure. The residue was washed with $CHCl_3$ (3 × 50 ml) to yield a partially purified catechin fraction as

amorphous brown solid (4.2 g). The above process was repeated several times to obtain the amount of partially purified catechin fraction needed for the experiments.

General Procedure for the Synthesis of O-*Acyl Derivatives of (−)-Epicatechin-3-gallate.* For the synthesis of the *O*-acyl derivatives of (−)-ECG, the partially purified catechin fraction was treated with various acylating agents (Ac_2O, propionic anhydride, butyric anhydride, isobutyric anhydride, and valeroyl chloride) in the presence of pyridine. After each reaction, the TLC analysis of the acylated material showed 4–5 spots. The acylated material was, therefore, subjected to the combined use of CC (SiO_2 (30 g/g of acylated mixture; 75 × 3 cm); $CHCl_3$/AcOEt gradient; fraction size, 50 ml) and prep. TLC to obtain the purified *O*-acylated (−)-ECG derivatives. The purity of these *O*-acyl derivatives was checked by HPLC and found to be >98%.

*(−)-5,7-*O-*Diacetyl-3′,4′-*O-*diacetyl-3″,4″,5″-triacetylepicatechin-3-*O-*gallate* (**2**). Semisolid (0.35 g, 0.47 mmol, 41.22%). R_f ($CHCl_3$/AcOEt 7:3) 0.45. $[\alpha]_D^{25} = -27.0$ ($c = 10.50$, $CHCl_3$). UV (MeOH): 266 (4.0), 228 (4.2). IR (KBr): 3498, 2941, 1764, 1726, 1621, 1597, 1496, 1437, 1372, 1190, 1131, 1098, 1049, 892, 790, 701, 669, 592. ^{1}H-NMR: 2.22–2.28 (overlapping *s*, 7 Me); 2.98 (*dd*, *J* = 2.2, 18.0, H_b–C(4)); 3.07 (*dd*, *J* = 4.6, 18.0, H_a–C(4)); 5.19 (br. *s*, H–C(2)); 5.62 (*m*, H–C(3)); 6.59 (*d*, *J* = 2.2, H–C(8)); 6.73 (*d*, *J* = 2.2, H–C(6)); 7.20 (*d*, *J* = 7.48, H–C(5′)); 7.28 (*d*, *J* = 1.94, H–C(6′)); 7.33 (*d*, *J* = 2.0, H–C(2′)); 7.61 (2*s*, H–C(2″), H–C(6″)). ^{13}C-NMR: 21.0–20.1 (7 Me); 25.9 (C(4)); 66.4 (C(3)); 76.4 (C(2)); 108.0 (C(8)); 108.8 (C(6)); 109.5 (C(4a)); 118.9 (C(2″), C(6″)); 120.8 (C(2′)); 123.3 (C(5′)); 124.1 (C(6′)); 130.3 (C(1″)); 135.1 (C(1′)); 141.5 (C(4″)); 142.7 (C(3′), C(4′)); 143.2 (C(3″), C(5″)); 149.7 (C(7)); 149.9 (C(5)); 154.7 (C(8a)); 163.6 (C=O); 166.4 (C=O); 167.3 (2 C=O); 168.1 (2 C=O); 168.5 (C=O); 170.2 (C=O). ESI-MS: 754.16 ($[M+NH_4]^+$).

*(−)-5,7-*O-*Dipropionyl-3′,4′-*O-*dipropionyl-3″,4″,5″-*O-*tripropionylepicatechin-3-*O-*gallate* (**3**). Semisolid (0.55 g, 0.65 mmol, 59.95%). R_f (hexane/AcOEt 7:3) 0.42. $[\alpha]_D^{25} = -31.81$ ($c = 11.0$, $CHCl_3$). UV (MeOH): 269 (3.4), 220 (4.2). IR (KBr): 3453, 2968, 1769, 1725, 1624, 1596, 1494, 1440, 1388, 1348, 1219, 1183, 1133, 1089, 918, 874, 837, 771, 681. ^{1}H-NMR: 1.21–1.28 (overlapping *s*, 7 Me); 2.49–2.62 (overlapping *s*, 7 CH_2); 2.89 (*dd*, *J* = 2.1, 17.8, H_b–C(4)); 2.95 (*dd*, *J* = 4.2, 17.7, H_a–C(4)); 5.10 (br. *s*, H–C(2)); 5.41 (*m*, H–C(3)); 6.56 (*d*, *J* = 2.2, H–C(8)); 6.67 (*d*, *J* = 2.2 Hz, H–C(6)); 6.59 (*d*, *J* = 7.62, H–C(5′)); 7.25 (*d*, *J* = 1.94, H–C(6′)); 7.33 (*d*, *J* = 2.0, H–C(2′)); 7.52 (2*s*, H–C(2″), H–C(6″)). ^{13}C-NMR: 9.2–8.7 (7 Me); 29.6–27.1 (7 CH_2); 25.9 (C(4)); 66.3 (C(3)); 76.5 (C(2)); 107.9 (C(8)); 108.7 (C(6)); 109.4 (C(4a)); 118.6 (C(2″), C(6″)); 121.1 (C(2′)); 123.1 (C(5′)); 124.2 (C(6′)); 134.4 (C(1″)); 135.4 (C(1′)); 141.2 (C(4″)); 142.4 (C(3′), C(4′)); 143.3 (C(3″), C(5″)); 149.6 (C(7)); 149.8 (C(5)); 154.7 (C(8a)); 163.1 (C=O); 170.2 (C=O); 171.1 (2 C=O); 171.9 (2 C=O); 172.5 (C=O); 173.9 (C=O). ESI-MS: 852.71 ($[M+NH_4]^+$).

*(−)-5,7-*O-*Dibutyryl-3′,4′-*O-*dibutyryl-3″,4″,5″-*O-*tributyrylepicatechin-3-*O-*gallate* (**4**). Semisolid (0.55 g, 0.59 mmol, 65.0%). R_f (hexane/AcOEt 8:2) 0.47. $[\alpha]_D^{25} = -20.0$ ($c = 10.0$, $CHCl_3$). UV (MeOH): 268 (4.3), 217 (4.3). IR (KBr): 3443, 2930, 1773, 1725, 1673, 1596, 1495, 1428, 1352, 1319, 1263, 1131, 1081, 900, 874, 770, 663. ^{1}H-NMR: 0.73–1.44 (overlapping *s*, 7 Me); 1.7–2.1 (overlapping *s*, 7 CH_2); 2.4–2.5 (overlapping *s*, 7 CH_2); 2.81 (*dd*, *J* = 1.5, 17.5, H_b–C(4)); 2.91 (*dd*, *J* = 4.3, 17.6, H_a–C(4)); 4.97 (br. *s*, H–C(2)); 5.37 (*m*, H–C(3)); 6.53 (*d*, *J* = 2.2, H–C(8)); 6.65 (*d*, *J* = 2.2, H–C(6)); 7.17 (*d*, *J* = 7.45, H–C(5′)); 7.26 (*d*, *J* = 2.0, H–C(6′)); 7.31 (*d*, *J* = 2.1, H–C(2′)); 7.61 (2*s*, H–C(2″), H–C(6″)). ^{13}C-NMR: 13.7–13.3 (7 CH_2); 18.4–18.2 (7 CH_2); 36.2–35.5 (7 Me); 26.0 (C(4)); 66.3 (C(3)); 76.5 (C(2)); 108.0 (C(8)); 108.8 (C(6)); 109.5 (C(4a)); 118.7 (C(2″), C(6″)); 121.8 (C(2′)); 123.1 (C(5′)); 124.0 (C(6′)); 134.1 (C(1″)); 135.5 (C(1′)); 141.0 (C(4″)); 142.0 (C(3′, 4′)); 143.3 (C(3″), C(5″)); 149.7 (C(7)); 149.9 (C(5)); 154.8 (C(8a)); 163.7 (C=O); 168.9 (C=O); 169.8 (2 C=O); 170.1 (2 C=O); 170.6 (C=O); 171.0 (C=O). ESI-MS: 950.38 ($[M+NH_4]^+$).

*(−)-5,7-*O-*Diisobutyryl-3′,4′-*O-*diisobutyryl-3″,4″,5″-*O-*triisobutyrylepicatechin-3-*O-*gallate* (**5**). White flakes (0.60 g, 0.64 mmol, 71.5%). M.p. 178–180°. R_f (hexane/AcOEt 8.5:1.5) 0.40. $[\alpha]_D^{25} = -27.8$ ($c = 10.4$, $CHCl_3$). UV (MeOH): 266 (2.4), 216 (3.2). IR (KBr): 3450, 2977, 2938, 1769, 1727, 1627, 1598, 1466, 1434, 1324, 1219, 1131, 1091, 909, 835, 770, 525. ^{1}H-NMR: 1.25–1.34 (overlapping *s*, 14 Me); 2.68–2.81 (overlapping *s*, 7 H–C); 2.83 (*dd*, *J* = 1.5, 17.5, H_b–C(4)); 2.92 (*dd*, *J* = 4.3, 17.6, H_a–C(4)); 5.11 (br. *s*, H–C(2)); 5.42 (*m*, H–C(3)); 6.50 (*d*, *J* = 2.2, H–C(8)); 6.67 (*d*, *J* = 2.2, H–C(6)); 7.16 (*d*, *J* = 7.52, H–C(5′)); 7.28 (*d*, *J* = 1.97, H–C(6′)); 7.32 (*d*, *J* = 2.1, H–C(2′)); 7.61 (2*s*, H–C(2″), H–C(6″)). ^{13}C-NMR: 19.0–18.5 (14 Me); 34.2–33.8 (7 CH); 25.9 (C(4)); 66.4 (C(3)); 76.4 (C(2)); 107.9 (C(8)); 108.7 (C(6));

109.4 (C(4a)); 118.5 (C(2″), C(6″)); 120.4 (C(2′)); 123.6 (C(5′)); 124.2 (C(6′)); 131.0 (C(1″)); 135.4 (C(1′)); 141.7 (C(4″)); 142.6 (C(3′), C(4′)); 143.6 (C(3″), C(5″)); 149.6 (C(7)); 149.9 (C(5)); 154.8 (C(8a)); 163.4 (C=O); 172.8 (C=O); 173.7 (2 C=O); 174.7 (2 C=O); 175.2 (C=O); 176.5 (C=O). ESI-MS: 950.38 ($[M+NH_4]^+$).

(−)-5,7-O-*Divaleroyl-3′,4′*-O-*divaleroyl-3″,4″,5″*-O-*trivaleroylepicatechin-3*-O-*gallate* (**6**). Semisolid (0.50 g, 0.48 mmol, 59.9%). R_f (hexane/AcOEt 9:1) 0.43. $[\alpha]_D^{25} = -30.7$ ($c = 10.4$, $CHCl_3$). UV (MeOH): 266 (3.2), 226 (4.1), 208 (4.2). IR (KBr): 3422, 2940, 2914, 2830, 1775, 1723, 1613, 1597, 1494, 1432, 1377, 1320, 1230, 1124, 1095, 1041, 900, 772, 658. ^{1}H-NMR: 0.91–0.95 (overlapping *s*, 7 Me); 1.35–1.39 (overlapping *s*, 7 CH_2); 1.63–1.71 (overlapping *s*, 7 CH_2); 2.41–2.50 (overlapping *s*, 7 CH_2); 2.95 (*dd*, J = 2.5, 17.7, H_b–C(4)); 2.98 (*dd*, J = 4.4, 18.1, H_a–C(4)); 5.09 (br. *s*, H–C(2)); 5.43 (*m*, H–C(3)); 6.49 (*d*, J = 2.2 Hz, H–C(8)); 6.65 (*d*, J = 2.2, H–C(6)); 7.15 (*d*, J = 7.48, H–C(5′)); 7.27 (*d*, J = 2.0, H–C(6′)); 7.30 (*d*, J = 2.0, H–C(2′)); 7.60 (2*s*, H–C(2″), H–C(6″)). ^{13}C-NMR: 13.6–13.5 (7 Me); 22.1–22.0 (7 CH_2); 26.8–26.6 (7 CH_2); 33.7–33.2 (7 CH_2); 26.0 (C(4)); 67.5 (C(3)); 76.4 (C(2)); 107.8 (C(8)); 108.8 (C(6)); 109.2 (C(4a)); 118.4 (C(2″), C(6″)); 120.5 (C(2′)); 123.2 (C(5′)); 124.4 (C(6′)); 130.8 (C(1″)); 142.4 (C(3′), C(4′)); 135.2 (C(1′)); 141.2 (C(4″)); 143.2 (C(3″), C(5″)); 149.6 (C(7)); 149.8 (C(5)); 154.7 (C(8a)); 163.4 (C=O); 170.4 (C=O); 171.8 (C=O); 172.3 (C=O); 173.5 (C=O); 174.8 (C=O). ESI-MS: 1048.49 ($[M+NH_4]^+$).

Animals. Female Swiss albino mice (LACCA, six-weeks-old) weighing 18–22 g were obtained from the Central Animal House of the Panjab University. The animals were kept in the Departmental Animal House with a controlled temp. of 23 ± 5°, 60 ± 5% humidity, and a 12 h light/dark cycle. They were fed a basal diet and H_2O. The mice were acclimatized for one week before experimentation. Animal care and handling was conducted according to the guidelines set by the *World Health Organisation* (*WHO*), Geneva, Switzerland, and the *Indian National Science Academy* (*INSA*), New Delhi, India. The protocol for the antitumor study was approved by the *Institutional Animal Ethics Committee.*

In vivo *Antitumor Activity.* Skin tumors were induced in Swiss albino mice (LACCA/female) according to the method of *Azuine* and *Bhide* [43]. Depilatory cream was used to remove the hairs from the back of the mice. The animals were divided into seven groups and given the following treatments 2 d after hair removal. The animals ($n = 10$) of the control group (*Group I*) were treated with 100 µl vehicle (acetone). The acetone was topically applied on the depilated back of each mouse twice weekly for 20 weeks. The animals of *Group II* ($n = 10$) were topically treated with 7,12-dimethylbenz[*a*]anthracene (DMBA; 100 nmol/100 µl acetone) on the depilated back of each mouse twice weekly for 2 weeks, followed by the topical application of 12-*O*-tetradecanoylphorbol-13-acetate (TPA; 17 nmol/100 µl acetone) twice weekly for the next 17 weeks. The animals of groups *III–VII* ($n = 10$) were orally treated with **2**–**6**, resp., suspended in H_2O/(methoxycarbonyl)cellulose (5%) at a dose of 50 mg/kg body weight. The dose of **2**–**6** was calculated on the basis of the ED_{50}, which was between 50 and 53 mg/kg body weight. The twice-a-week treatment with **2**–**6** started one week before the topical application of DMBA (100 nmol/100 µl acetone) and was continued for the next 19 weeks. The cancer induction and promotion were achieved as described for the animals of *Group II*. The body weight, the incidence of skin papillomas, and the number of surviving animals were recorded weekly. Only those papillomas that persisted for 2 weeks or more were taken into consideration for the final evaluation of the data.

Histology of Papillomas. The skin papillomas and the normal skin tissue were fixed in *Zenker*'s fixative fluid, routinely processed, and embedded in paraffin. Sections (7 µm) were stained with hematozylin and eosin and examined under a light microscope.

Nuclear Extract Preparation and Protein Estimation by ELISA. The nuclear extract was prepared by homogenizing the tumor and control skin (10%) in 2 ml of *Buffer A* (50 mM NaCl, 10 mM *HEPES* pH 8, 500 mM sucrose, and 1 mM EDTA). After homogenizing, 0.2% *Triton-X 100* and 1 mM phenylmethylsulfonylfluoride (PMSF) were added. This mixture was then centrifuged at 5,000 rpm for 2 min under cold conditions. The supernatant (cytoplasmic extract) was removed, and the pellet was resuspended in 500 µl of *Buffer B* (50 mM NaCl, 10 mM *HEPES* pH 8, 25% glycerol, 1 mM EDTA, and 1 mM PMSF). This mixture was then centrifuged at 5,000 rpm for 3 min under cold conditions. The supernatant was discarded, and the pellet was resuspended in 50 µl of *Buffer C* (350 mM NaCl, 10 mM *HEPES* pH 8, 25% glycerol, 1 mM EDTA, and 1 mM PMSF), incubated on ice for 30 min with constant shaking, and then centrifuged at 10,000 rpm for 10 min under cold conditions. The supernatant (nuclear

extract) was removed and used for further analyses. The protein concentration in the nuclear extract was determined using the method described by *Bickers* and *Lowy* [44]. ELISA was carried out to quantify the concentration of c-Jun, p65, and p53 in the nuclear extract of control skin and tumors. The assay was standardized by titration of the different concentrations of antigens and antibodies. The wells were coated with 5 μg of antibodies for c-Jun, p65, and p53 proteins in 100 μl of 0.05M carbonate buffer (pH 9.6), and the plates were kept overnight at 4° in a moist chamber. The plates were flicked to remove the unbound antigen/antibody soln., and the wells were blocked with 1% BSA in 0.1M phosphate buffered saline (PBS pH 7.2) for 1 h at 37°. The plates were flicked, and the wells washed thrice with 200 μl of PBS containing 0.05% (*v/v*) *Tween-20*. The wells were then incubated with anti-c-Jun (1:250), anti-p65 (1:1000), anti-p53 (1:300), and primary antibody, resp., diluted in PBS containing 0.05% *Tween-20* and 1% BSA, and kept for 2 h at 37°. The plates were washed again, and the wells were incubated with peroxidase labeled anti-rabbit secondary antibody (*Sigma-Aldrich*) 1:1000 for c-Jun and p65 and 1:300 for p53. The wells were incubated with anti-sheep IgG (1:1000; *Sigma-Aldrich*) for 2 h at 37° and further washed three times as described above. The color was developed by addition of 2,20-azinodi(3-ethyl)benzothiozolinsulfonic acid reagent, and the absorbance at 405 nm was measured by the ELISA reader.

HPLC Analysis of the O*-Acyl Derivatives of (−)-Epicatechin-3-gallate.* The *O*-acyl derivatives of (−)-ECG, **2**–**6**, were quantified by isocratic reversed-phase (RP) HPLC using a *Waters* equipment consisting of two *501* pumps controlled by an automated gradient controller, a *Waters 481* UV detector, a *746* data module, a 20 μl *Rheodyne* loop injection valve, and a RP *LiChrosphere* C_{18} column (250 × 4.6 mm; 10 μm particles) eluted with mixtures of MeOH or MeCN and 0.02M phosphate buffer (pH 3.5–4.5). The composition of the eluent and pH of the buffer were adjusted for each compound in order to provide an appropriate t_R and separation of the *O*-acyl derivatives of (−)-ECG. A flow rate of 1 ml/min was maintained, and the column effluent was monitored at 270 nm. Quantification of the compounds was carried out by measuring the peak areas or peak heights in relation to those of standard **1** chromatographed under identical conditions.

Solubility Determination. The Solubility of **2**–**6** was determined by HPLC in 0.05M phosphate buffer pH 7.4 at 25° using a H_2O-bath shaker [45]. An excess amount of each compound was added to buffer (2.0 ml) in screw-capped test tubes and the suspensions vortexed for 10 min and shaken for 24 h at 25° in a H_2O-bath shaker. The solns. were filtered through membrane filters (0.45 μm) in a warm test tube, and after appropriate dilution in the same buffer, aliquots of 10 μl were injected. The concentration of each compound was calculated from the standard plot obtained the same day under similar conditions. Determinations were performed in triplicate for each compound.

Lipophilicity Determination. The apparent partition coefficients (*P*) of **2**–**6** were determined in octanol/0.05M phosphate buffer pH 7.4 at 25° [35], using the shake-flask method [46]. The phosphate buffer and octanol were mutually saturated by shaking overnight. The system was left undisturbed for 30 min, and the layers were separated. Saturated solns. of the compounds were prepared in octan-1-ol (2 ml) and phosphate buffer (5 ml) was added to the solns. in conical flasks. The sealed flasks were kept shaking in a H_2O-bath shaker maintained at 25 ± 2° for 8 h. The octanol layer was removed and appropriately diluted. The amount of drug partitioned into the buffer and octanol layers was analyzed by HPLC by injecting 10 μl of each layer. The concentration was determined by HPLC to afford rapid evaluation and better reliability. The partition coefficients were calculated with *Eqn. 1*, and the values were reported as log *P:*

$$\text{Partition coefficient } (P) = \text{AUC}_{\text{octanol}}/\text{AUC}_{\text{buffer}} \times \text{dilution factor} \quad (1)$$

Chemical Hydrolysis. The chemical stability of the (−)-ECG derivatives **2**–**6** in isotonic phosphate buffer pH 7.4 and in HCl buffer pH 2.0 was studied at 37°. The reaction was initiated by adding 20–50 μl of the stock soln. (**2**, 1.8 mg/10 ml; **3**, 1.3 mg/10 ml; **4**, 1.1 mg/10 ml; **5**, 12 mg/10 ml; **6**, 1.4 mg/10 ml) of each derivative diluted in MeCN to 2–5 ml of preheated buffer soln. in a screw-capped test tube. The solns. were kept in a H_2O bath at constant temp. and samples were withdrawn at appropriate time intervals and analyzed by HPLC for the appearance of (−)-ECG (**1**). Pseudo first-order rate constants (k_{obs}) for the hydrolysis of the derivatives were determined from the slopes of the linear plots of the

logarithms of residual derivative against time (*Eqn. 2*). The half-lives ($t_{1/2}$) were determined from the pseudo-first-order rate constants using *Eqn. 3*.

$$k_{obs} = \text{slope} \times 2.303 \quad (2)$$

$$t_{1/2} = 0.693/k_{obs} \quad (3)$$

Enzymatic Hydrolysis. The enzymatic hydrolysis of the (−)-ECG derivatives **2**–**6** was studied in human plasma (80%) at pH 7.4. The reaction was initiated by adding 20–50 µl of the stock soln. (see above) of the derivative in MeCN to 2–5 ml of preheated plasma soln. The soln. was kept in a H_2O bath at 37°, and samples of 100–250 µl were withdrawn at appropriate time intervals and added to 1–5 ml of cold MeCN or MeOH to precipitate the plasma proteins. After immediate mixing and centrifugation for 5 min at 7000 rpm, 20 µl of the clear supernatants were analyzed by HPLC for the appearance of compound **1**. The rate constant (k_{obs}) and half-lives ($t_{1/2}$) were determined as described above (see *Chemical Hydrolysis*).

Statistical Analysis. The data were analyzed by analysis of variance (ANOVA), *Student*'s *t*-test, and the χ^2 test. A statistical value of $P < 0.05$ was considered significant.

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