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Mechanism of biochemical action of substituted 4-methylcoumarins. Part 11: Comparison of the specificities of acetoxy derivatives of 4-methylcoumarin and 4-phenylcoumarin to acetoxycoumarins: protein transacetylase

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Abstract—Our earlier observations led to the identification of a microsomal enzyme termed as acetoxy drug: protein transacetylase (TAase) catalyzing the transfer of acetyl groups from acetylated polyphenols to the receptor proteins. TAase was conveniently assayed by the irreversible inhibition of cytosolic glutathione S-transferase (GST) by the model acetoxycoumarin, 7,8-diacetoxy-4-methylcoumarin (**1**). The specificities of the acetoxy group on the benzenoid ring and position of the pyran carbonyl group of the coumarin with respect to oxygen heteroatom for the catalytic activity of TAase were also reported earlier. In this communication, we have demonstrated that the acetoxy coumarins and acetoxy dihydrocoumarins having a methyl group instead of a phenyl ring at the C-4, when used as the substrates, resulted in enhancement of TAase activity, while the saturation of double bond at C-3 and C-4 position had no effect on TAase activity. A comparison of the optimized structures of **1** and 7,8-diacetoxy-4-phenylcoumarin (**2**) suggested that the observed influence may be due to out of plane configuration of the phenyl ring at C-4. Further, the TAase-catalyzed activation of NADPH cytochrome c reductase and inhibition of aflatoxin B_1 (AFB_1)–DNA binding by acetoxy 4-phenylcoumarins and dihydrocoumarins were significantly lower as compared to those caused by acetoxy 4-methylcoumarins.

1. Introduction

The presence of a novel enzyme in microsomes of rat liver catalyzing the possible transfer of acetyl groups from acetylated polyphenols to certain receptor enzyme proteins resulting in the modification of their catalytic activities was documented in earlier communications from our laboratory. The enzyme was termed as acetoxy drug (e.g., acetoxycoumarins): protein transacetylase.[1–3] Recently, we reported the mass spectrometric evidence for the acetylation of the glutathione S-transferase (GST 3-3) by the diacetoxycoumarin **1** catalyzed by a partially purified TAase.[4,5] The need for understanding the specificity of acetoxycoumarins for TAase has necessitated the quantitative structure–activity relationship (QSAR) studies. We have earlier reported certain structural features of acetoxycoumarins specific for TAase activity.[3] In this communication, we have demonstrated that acetoxycoumarins having a phenyl moiety at the C-4 position instead of a methyl group are poor substrates for the TAase enzyme.

2. Results

In this report, we have meticulously compared the specificities of acetoxy derivatives of a large number of

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4-methylcoumarin, 4-methyldihydrocoumarin, 4-phenylcoumarin, and 4-phenyldihydrocoumarin in order to bring out clearly the effect of the replacement of methyl group (at C-4) by a phenyl moiety. For this purpose, a number of acetoxy coumarins and dihydrocoumarins bearing a phenyl ring and methyl group at C-4 were synthesized (Fig. 1). The results (Fig. 2) clearly indicate that 4-methyl-7,8-diacetoxycoumarin (**1**) shows the highest catalytic activity for the TAase in comparison to **2** and other acetoxycoumarins, although both compounds **1** and **2** have a double bond between C-3 and C-4 positions. It is also evident from Figure 2 that activities of diacetoxy 4-methylcourmarins are twice as high as those of the corresponding diacetoxy

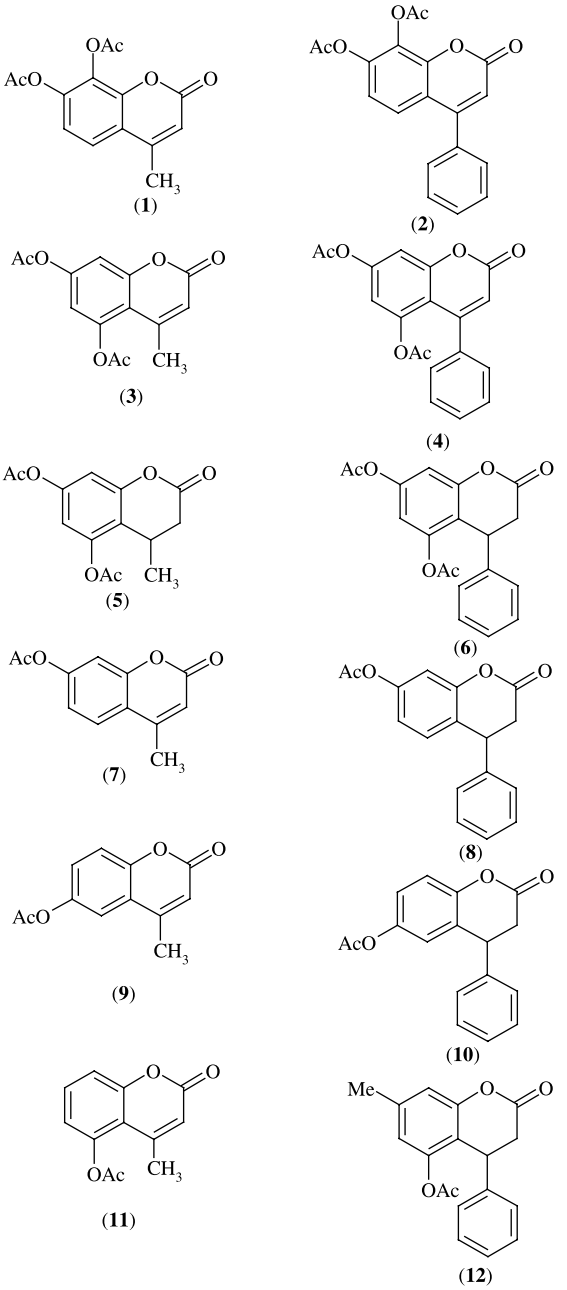

Figure 1. The structures of acetoxycoumarins. Numbers in parenthesis denote compound number.

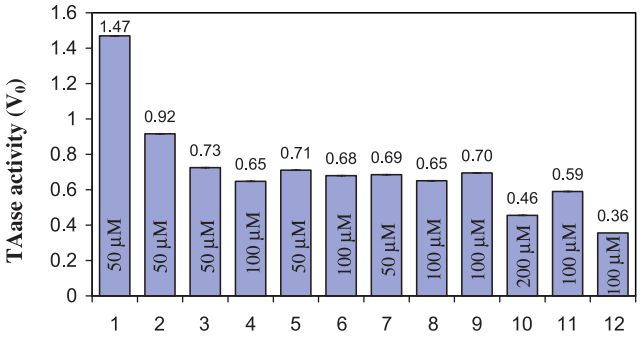

Figure 2. Comparison of the specificities of the acetoxy derivatives of 4-methylcoumarin and 4-phenylcoumarin to acetoxycoumarins: protein transacetylase. V_0 = initial rate of TAase activity (units/min of preincubation) indicated on the top of the bar. Concentration of the test compounds indicated inside the bar. Units of TAase activity expressed in terms of the % inhibition of GST under conditions of the assay. Values are mean of three observations with variation less than 5%.

4-phenylcoumarins, that is, the activity of compound **4** can be simulated to that of compound **3** by doubling the concentration of **4**. Interestingly, such a trend also persisted for the diacetoxy dihydrocoumarin analogs **5** and **6**, that is, the 4-methyl analog **5** is as active as the 4-phenyldihydrocoumarin **6** at just half the concentration of **6** (Fig. 2).

While comparing the activities of coumarins with those of the corresponding dihydro analogs, no significant change in the TAase activity was observed, that is, the 5,7-diacetoxy-4-methylcoumarin (**3**) and its dihydro analog **5** have similar activity. Similarly the activity of 5,7-diacetoxy-4-phenylcoumarin (**4**) is nearly same as that of its dihydro analog **6**. Also, the TAase activities of monoacetoxy 4-methylcoumarins **7**, **9**, and **11** were compared with those of the corresponding 4-phenyldihydrocoumarins **8**, **10**, and **12**, it was observed (Fig. 2) that **7** is twice as active as **8**. Similar trends were found in case of the pairs of compounds **9** and **10**, and **11** and **12**.

The TAase catalyzed activation of NADPH cytochrome c reductase by acetoxy 4-methylcoumarins was compared with the acetoxy derivatives of 4-phenylcoumarins and dihydrocoumarins, the results shown in Figure 3 revealed that 4-methyl-7,8-diacetoxycoumarin (**1**) upon incubation with liver microsomes activated the reductase to the maximum extent. The monoacetoxy 4-methylcoumarins **7** and **9** caused significantly higher activation of the reductase as compared to their 4-phenyldihydrocoumarin analogs **8** and **10**, respectively. In general, the acetoxy 4-phenylcoumarins were found to activate the reductase to significantly lesser extent than the acetoxy 4-methylcoumarin counterparts. The abilities of acetoxy 4-methylcoumarins and acetoxy 4-phenylcoumarins to inhibit liver microsomal catalyzed AFB_1 binding to DNA are compared in Figure 4. Pattern of inhibition of AFB_1–DNA binding by acetoxy derivatives of 4-methyl- and 4-phenyldihydrocoumarins followed similar trend as observed earlier. Accordingly,

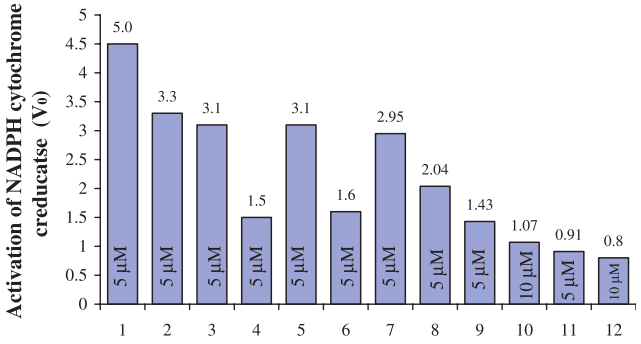

Figure 3. TAase catalyzed irreversible activation of NADPH cytochrome c reductase by acetoxycoumarins: protein transacetylase. V_0 = initial rate (percent activation/min of preincubation) indicated on the top of the bar. Concentration of the test compounds inside the bar. Values are mean of three observations with variations less than 2%.

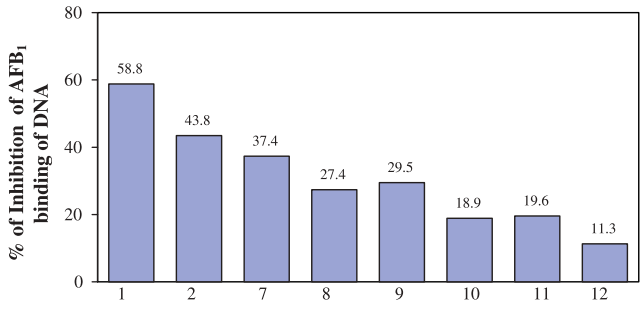

Figure 4. Comparison of TAase catalyzed modulation of rat liver microsomes mediated aflatoxin B_1 binding to DNA in vitro by acetoxy derivatives of 4-methyl, 4-phenyl, and 4-phenyldihydrocoumarins. Inhibition of AFB_1 binding to DNA (Pmoles/mg/30 min) indicated on the top of the bar. Concentration of the test compounds was 100 µM. Values are mean of three observations with variations less than 5%.

acetoxy-4-phenyldihydrocoumarins inhibited liver microsomal cytochrome P-450 catalyzed AFB_1 epoxidation (measured as binding to DNA) to a significantly lesser extent than the acetoxy derivatives of 4-methylcoumarins (cf. the pairs **7** and **8**; **9** and **10**; **11** and **12**, Fig. 4).

The PM3 optimized geometries have revealed that the C-8 acetoxy groups in 7,8-diacetoxycoumarin are bent more toward the oxygen atom of the pyran ring. This is true for both 4-phenyl and 4-methyl derivatives of 7,8-diacetoxycoumarins (Figs. 5 and 6), the geometry and the charge distribution in 4-phenyl- and 4-methyl-7,8-diacetoxycoumarin are nearly similar. However, the optimized geometry of 4-phenyl-7,8-diacetoxycoumarin shows an out of plane orientation of the phenyl ring at C-4 (Fig. 6, degree of non-planarity = −58.1).

3. Discussion

The existence of an unique enzyme termed as acetoxy drug: protein transacetylase (TAase) in rat liver microsomes catalyzing transfer of the acetyl group from acetoxy xenobiotics to specific proteins was evidenced from our studies published earlier.[2,3,24] We have recently conclusively established TAase catalyzed transfer of acetyl groups from a model acetyl donor **1** to a receptor protein (GST 3-3) as evidenced by mass spectrometry studies.[4,5] As a part of the quantitative structure–activity relationship (QSAR) studies on TAase, we have demonstrated (a) positional specificity of acetoxy groups on the benzenoid ring of certain polyphenols[3] and (b) absolute requirement of carbonyl group on pyran ring.[24] We have in this report highlighted the outcome of the replacement of the C-4 methyl group by a phenyl group in the pyran ring of acetoxycoumarins on the catalytic activity of TAase. For this task, a number of acetoxy 4-phenylcoumarins and dihydrocoumarins were synthesized and their specificities to TAase were compared with those of the acetoxy 4-methylcoumarins and dihydrocoumarins. The pairs of compounds **1**–**2**, **3**–**4**, and **5**–**6** (Fig. 1) only differ in substitution at the C-4 position of pyran ring, that is, in these pairs of compounds, the former have a C-4 methyl group and the latter have a C-4 phenyl group. Higher TAase activities were found in all the compounds with methyl group at the C-4 position (Figs. 2–4), it becomes evident that the compounds having a methyl group at the C-4 position have higher TAase activity in comparison to compounds having C-4 phenyl group. However, the TAase activities of **3** and **5** are in close proximity (Figs. 2 and 3), a similar trend persisted with compounds **4** and **6**. These results convincingly suggest that the unsaturation at C-3 and C-4 had no effect on TAase activity, while the substitution of methyl group by phenyl group accounted for significant increase in inhibition of TAase activity. These results ruled out any role of C-3, C-4 double bond in the pyran ring of coumarins on their abilities to transfer acetyl group to the functional proteins. The optimized geometries of these compounds show that the phenyl group at C-4 position, unlike the methyl group acquires

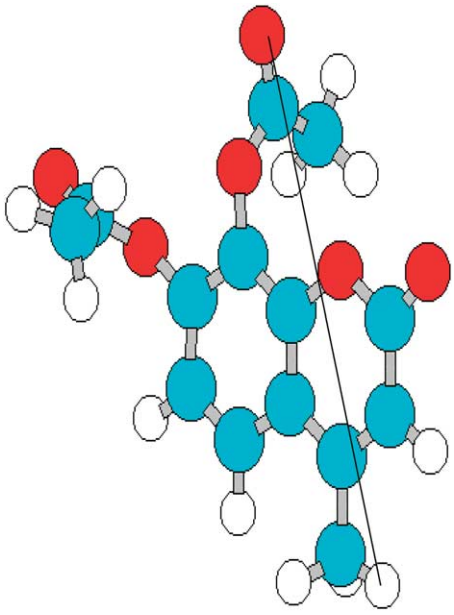

Figure 5. Optimized molecular structure of 7,8-diacetoxy-4-methylcoumarin (**1**) as obtained by PM3 calculations.

an out of plane orientation (cf. pairs of Figs. 5 and 6; 7 and 8; and 9 and 10). Further the longest axis measurement (11.872 Å) in the optimized geometry of 7,8-diacetoxy-4-phenylcoumarin (**2**) (Fig. 6) is found to be approximately 1.4 times greater than that in 7,8-diacetoxy-4-methylcoumarin (**1**) (8.650 Å) (Fig. 5). These results are suggestive of hindrance for the acetoxy group from accessing the active site of TAase resulting in the decreased rate of transfer of the acetyl group to the receptor protein in the case of **2** as compared to **1**. PM3 bond orders calculated between the oxygen atom (O–) and the carbon atom (C–) of the acetyl group in 5,7-diacetoxy-4-methylcoumarin and 5,7-diacetoxy-4-phenylcoumarin derivatives are almost similar to their respective dihydro derivatives. This is in agreement with observed fact that the TAase catalytic activities of 5,7-diacetoxy-4-methylcoumarin (**3**) and 5,7-diacetoxy-4-

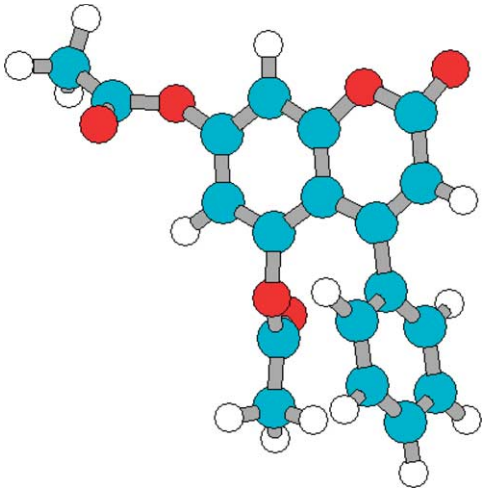

Figure 8. Optimized molecular structure of 5,7-diacetoxy-4-phenylcoumarin (**4**) as obtained by PM3 calculations.

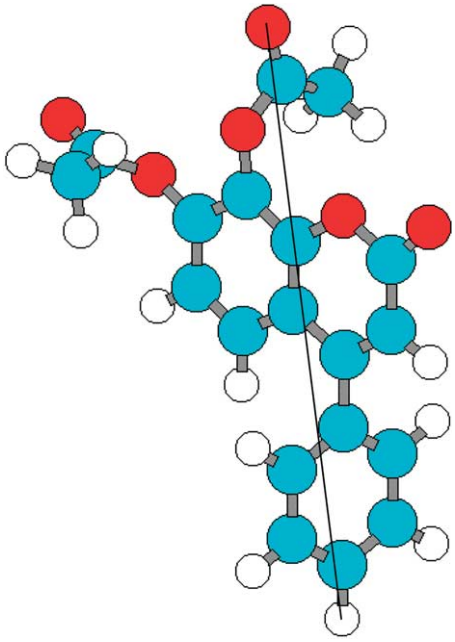

Figure 6. Optimized molecular structure of 7,8-diacetoxy-4-phenylcoumarin (**2**) as obtained by PM3 calculations.

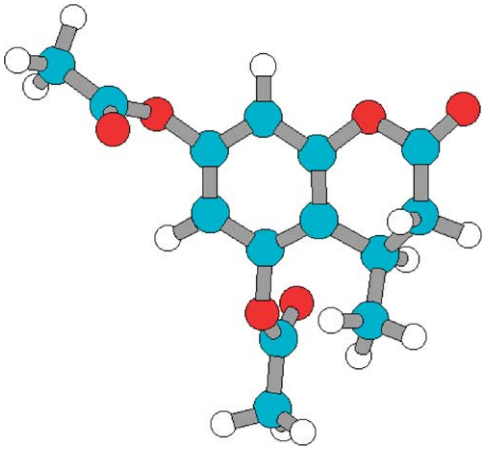

Figure 9. Optimized molecular structure of 5,7-diacetoxy-4-methyl-3,4-dihydrocoumarin (**5**) as obtained by PM3 calculations.

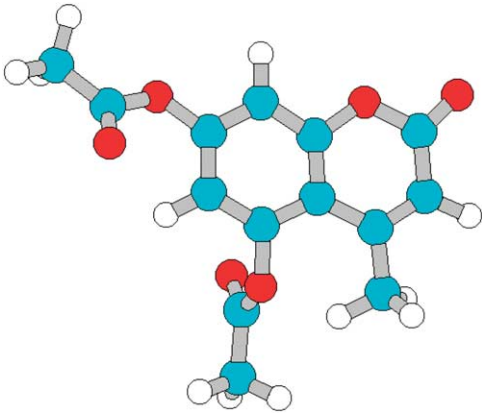

Figure 7. Optimized molecular structure of 5,7-diacetoxy-4-methylcoumarin (**3**) as obtained by PM3 calculations.

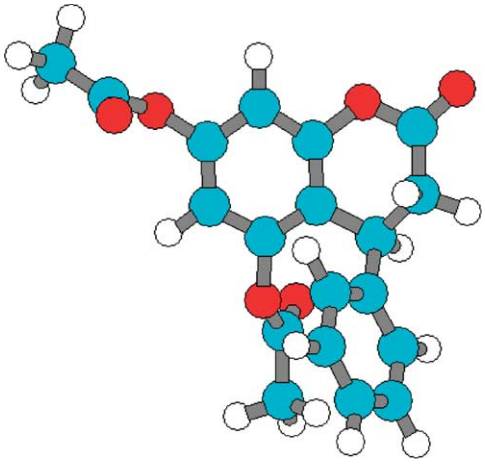

Figure 10. Optimized molecular structure of 5,7-diacetoxy-4-phenyl-3,4-dihydrocoumarin (**6**) as obtained by PM3 calculations.

phenylcoumarin (**4**) are similar to those of their respective dihydro analogs **5** and **6**, respectively (Figs. 2 and 3). However, the low activity of 4-phenyl derivatives as compared to those of the corresponding 4-methyl counterparts can be attributed to the predominant steric factors arising from the non-planar conformation of the phenyl ring at the C-4 position (Figs. 6, 8 and 10).

The irreversible activation of NADPH cytochrome c reductase and the inhibition of cytochrome P-450 linked Mixed Function Oxidases by **1** were established as the TAase related biological effects.[1,2] As observed earlier with other polyphenolic acetates,[3,24] the acetoxy derivatives of 4-phenylcoumarin and dihydrocoumarin modulate reductase and cytochrome P-450 activities (microsome mediated AFB_1 epoxidation) in a manner proportional to their specificity to TAase. Accordingly, TAase catalyzed activation of reductase and inhibition of AFB_1–DNA binding by 4-phenylcoumarins and dihydrocoumarins were found to be significantly lower than those by the 4-methylcoumarin counterparts.

4. Experimental

4.1. Materials and methods

4.1.1. Chemicals. The organic solvents (acetone, acetic anhydride, chloroform, and pyridine) used were dried and distilled prior to their use. Analytical TLCs were performed on precoated Merck silica gel 60 F_{254} plates; the spots were visualized under UV light. The ^1H and ^{13}C NMR spectra were recorded on a Brucker AC-300 Avance spectrometer at 300 and 75.5 MHz, respectively, using TMS as internal standard. The chemical shift values are on δ scale and the coupling constant values (J) are in hertz. NADPH, Cytochrome c, reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), and calf thymus DNA were obtained from Sigma Chemical Company, St. Louis, MO (USA). [^3H] AFB_1 was purchased from Moravek Biochemicals, Brea, CA (USA).

4.1.2. Synthesis of coumarins. We have synthesized twelve coumarins (Fig. 1) having various substituents at different positions by using literature procedures with some modifications, 5,7-diacetoxy-4-methyl-3,4-dihydrocourmarin (**5**) is a novel compound and has been synthesized for the first time. 7,8-Dihydroxy-4-methylcoumarin was prepared by Pechman condensation[6] of pyrogallol with ethyl acetoacetate in the presence of H_2SO_4; its diacetoxy derivative, 7,8-diacetoxy-4-methylcoumarin (**1**) was obtained by its acetylation with acetic anhydride–pyridine in the presence of catalytic amount of DMAP. Similarly, condensation of pyrogallol with ethyl benzoylacetate in H_3PO_4 yielded 7,8-dihydroxy-4-phenylcoumarin, its subsequent acetylation afforded 7,8-diacetoxy-4-phenylcoumarin (**2**).[7,8] The compound 5,7-diacetoxy-4-methylcoumarin (**3**) was obtained by the acetylation of 5,7-dihydroxy-4-methylcoumarin.[9] 5,7-Diacetoxy-4-phenylcoumarin (**4**) was prepared by acetylating the condensation product of phloroglucinol and ethyl benzoylacetate[10] in the presence of trifluoroacetic acid (TFA) and its dihydro analog 5,7-diacetoxy-4-phenyl-3,4-dihydrocoumarin (**6**) was synthesized by the method described in literature[11] using Michael addition of phloroglucinol to meldrum's acid and benzaldehyde. Again, Pechman condensation[6] of resorcinol and ethyl acetoacetate, followed by acetylation was used to synthesize 7-acetoxy-4-methylcoumarin (**7**). The acetoxy dihydrocoumarins, that is, 7-acetoxy-4-phenyl-3,4-dihydrocoumarin (**8**), 6-acetoxy-4-phenyl-3,4-dihydrocoumarin (**10**), and 5-acetoxy-7-methyl-4-phenyl-3,4-dihydrocoumarin (**12**) were synthesized by our earlier reported procedure.[12] 6-Acetoxy-4-methylcoumarin (**9**) and 5-acetoxy-4-methylcoumarin (**11**) were prepared by methods reported in the literature.[13,14] All the compounds described above were fully characterized by their melting point, UV, IR, and NMR data and by comparison with the data reported in the literature.

4.1.3. 5,7-Diacetoxy-4-methyl-3,4-dihydrocoumarin (5). Phloroglucinol (630 mg, 5 mmol) and meldrum's acid (720 mg, 5 mmol)[15] were dissolved in pyridine (30 ml) and nitrogen was bubbled through the solution for 10 min. Acetaldehyde (240 mg, 5.5 mmol) was added to it and the mixture stirred at 70–80 °C for 1 h. After cooling to room temperature, the reaction mixture was poured into ice water, extracted with ethyl acetate, organic layer dried, and evaporated under vacuum. The residue was purified by column chromatography using acetone–chloroform (7:93) to furnish 5,7-dihydroxy-4-methyl-3,4-dihydrocoumarin in 50% yield. IR (KBr): 3392 (OH), 1634 (CO), 1467, 1251, and 1139 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 0.98 (3H, br s, CH_3), 2.45–2.49 (1H, m, C–4H), 2.79–2.83 (2H, m, C–3H), 5.86 (1H, s, C–6H), 6.07 (1H, s C–8H), 9.36 (1H, s, OH), and 9.63 (1H, s, OH); ^{13}C NMR: (75.5 MHz, DMSO-d_6): δ 20.41 (CH_3), 23.97 (C-4), 36.44 (C-3), 94.94 (C-6), 98.92 (C-8), 105.97 (C-10), 152.45 (C-9), 155.29 (C-7), 157.55 (C-5), and 168.64 (C-2); EI-HRMS: m/z 194.0562 $[M]^+$, $C_{10}H_{10}O_4$, calcd 194.0579. The compound so obtained was treated with acetic anhydride in pyridine (anhydrous) containing a catalytic amount of 4-*N*,*N*-dimethylaminopyridine (DMAP), reaction was monitored on TLC, and after completion, the reaction mixture was poured into ice-cold water. Extraction with chloroform and recrystallization of the crude product thus obtained, furnished **5** as a viscous oil in 90% yield. IR (KBr): 1772 ($OCOCH_3$), 1624 (CO), 1431, 1371, 1199, 1122, 1087, and 1049 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 1.21 (3H, d, J = 7.12 Hz, CH_3), 2.28 (3H, s, $COOCH_3$), 2.32 (3H, s, $COOCH_3$), 2.74–2.77 (2H, m, C–3H), 3.20–3.32 (1H, m, C–4H), and 6.76 (2H, s, C–6H and C–8H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 20.20 (CH_3), 21.16 and 21.39 ($COOCH_3 \times 2$), 25.76 (C-4), 36.22 (C-3), 109.08 (C-6), 112.76 (C-8), 118.56 (C-10), 148.07 (C-7), 150.34 (C-5), 152.40 (C-9), 167.31 (C-2), and 168.99 ($COOCH_3 \times 2$); EI-HRMS: m/z 278.0795 $[M]^+$, $C_{14}H_{14}O_6$, calcd 278.0790.

4.1.4. Assay of acetoxy coumarin: protein transacetylase (TAase). TAase activity was assayed in rat liver microsomes. Preparation of rat liver microsomes and cytosol was described in our earlier communication.[16] TAase assay using acetoxycoumarins (AC) and cytosolic GST as the substrate was elaborated in detail earlier.[3] Briefly,

the assay mixture consisted of 0.25 M potassium phosphate buffer (pH 6.5), liver microsomes (25 μg protein), AC (concentration as in Fig. 2), liver cytosol (10–15 μg protein), and water to make up the total volume of 0.8 ml. The contents of the tube (scaled up as per the requirement) were preincubated for 10 min at 37 °C. The aliquots (0.8 ml portion) were removed periodically into spectrophotometer cuvette containing CDNB and GSH to make the final concentration 1 mM in a total volume of 1 ml and GST activity was assayed by monitoring absorption at 340 nm as described by Habig et al.[17] The unit of TAase catalyzed activity was expressed in terms of percent inhibition of GST activity under the conditions of the assay procedure.

4.1.5. TAase mediated biochemical action of acetoxycoumarins (AC). The rat liver microsomes (40 μg protein) were incubated with AC (concentration as in Fig. 3), 0.05 M phosphate buffer (pH 7.7), and water to make upto 0.5 ml. The contents (scaled up as per requirement) were preincubated at 37 °C in a shaking water bath. The aliquots (0.5 ml portion) were removed periodically into spectrophotometer cuvette containing 0.1 mM EDTA, 36 mM cytochrome c, and 1 mM NADPH in a total volume of 1 ml. The progress of NADPH cytochrome c reductase assay was followed by monitoring the absorption at 550 nm.[18] In the control samples, DMSO replaced AC. The increment in reductase activity due to AC over the control was expressed as percent activation. Liver microsome catalyzed inhibition of AFB_1 epoxidation (measured as binding to DNA) in vitro by AC was carried out as described in our earlier communication.[1]

4.1.6. Optimization of structures of 7,8-diacetoxy-4-methylcoumarin (1), 7,8-diacetoxy-4-phenylcoumarin (2), 5,7-diacetoxy-4-methylcoumarin (3), 5,7-diacetoxy-4-phenylcoumarin (4), 5,7-diacetoxy-4-methyl-3,4-dihydrocoumarin (5), and 5,7-diacetoxy-4-phenyl-3,4-dihydrocoumarin (6). The initial geometries of all the systems were obtained by HyperChem™ 5.1 Pro (Hypercube Inc., USA), which were optimized by the molecular mechanics (MM+) force field method using Polak–Ribiere (conjugate gradient) algorithm. All final geometry optimizations were performed using the PM3[19,20] Hamiltonian through the eigenvector following (EF) routine[21] on restricted Hartree–Fock basis without any conformational or symmetry restrictions. As most of the biochemical processes occur in aqueous medium, macroscopic solvent effects on the 4-substituted 5,7-diacetoxycoumarins were investigated within the framework of Continuum Solvation model using the COSMO (Conductor like Screening Model)[22] method as implemented in MOPAC 97 in the CS MOPAC Pro version 5.0, with dielectric constant taken as 78.5 for water at 298 K. In this model, the solute molecule is embedded in a cavity constructed from the overlapping van der Waals spheres[23] of the component atoms surrounded by a dielectric continuum of permitivity ε. This is a good approximation for solvents of high relative permitivity, such as water.

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