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Original article

Specificities of acetoxy derivatives of coumarins, biscoumarins, chromones, flavones, isoflavones and xanthones for acetoxy drug: Protein transacetylase

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Abstract

The earlier work carried out in our laboratory led to the identification of a novel rat liver microsomal enzyme termed as acetoxy drug: protein transacetylase (TAase), catalyzing the transfer of acetyl group from polyphenolic acetates (PA) to functional proteins. In this paper, we have reported the comparison of the specificities of acetoxy derivatives of coumarins, biscoumarins, chromones, flavones, isoflavones and xanthones with special reference to the phenyl moiety/bulky group on the pyran ring of PA. The results clearly indicated that compounds having phenyl moieties, when used as the substrates, resulted in a significant reduction of TAase catalyzed activity. The alteration in TAase catalyzed activation of NADPH cytochrome *c* reductase and inhibition of benzene-induced micronuclei in bone marrow cells by PA were in tune with their specificities to TAase.

Keywords: Polyphenolic acetates; Glutathione *S*-transferase (GST); Micronuclei modulation; NADPH cytochrome *c* reductase; Protein transacetylase (TAase)

1. Introduction

Acetoxy drug: protein transacetylase (TAase) is a unique enzyme bound to microsomal membrane of several tissues [1]. TAase was found to catalyze the transfer of acetyl group from 7,8-diacetoxy-4-methylcoumarin (**1**), a model acetoxy drug, to glutathione *S*-transferase (GST) resulting in the acetylation of several lysine residues in its active site and subsequently to inhibition of catalytic activity of GST [2,3]. The coumarin **1** was also found to modulate the activities of liver microsomal cytochrome P-450 catalyzed mixed function oxidases (MFO) [4] and NADPH cytochrome *c* reductase [5] catalyzed by TAase, possibly by way of acetylation of these enzyme proteins. Later, a number of acetoxy derivatives of coumarins and flavones were examined for their specificity to liver microsomal TAase. These investigations highlighted the structural features of polyphenolic acetates such as the proximity of acetoxy groups to the oxygen heteroatom, cardinal role of carbonyl group, and the influence of phenyl group substitution on the pyran ring, in controlling their specificities for the TAase [6,7]. In the present investigation, an effort has been made to compare the specificities of acetates of various polyphenols such as coumarins, biscoumarins, chromones,

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flavones, isoflavones and xanthones to liver microsomal TAase in order to delineate the structure–activity relationship (SAR) with special reference to the effect of phenyl ring on the pyran moiety of the polyphenolic acetates. The results clearly demonstrated that the specificity of various polyphenolic acetates for TAase is in the order: acetoxycoumarins = acetoxychromones > acetoxyflavones = acetoxy xanthones. It can be concluded that the substitution by phenyl moiety/bulky group at any position of pyran nucleus of the polyphenolic acetates leads to diminished affinity for TAase.

2. Chemistry

2.1. Chemicals

The organic solvents (acetone, acetic anhydride, chloroform and pyridine) 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 ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance 300 MHz NMR spectrometer at 300 MHz 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 Hz. Melting points were determined in a sulfuric acid bath and are uncorrected. The UV and IR spectra were recorded on a Cary Bio 100 and Perkin–Elmer model 2000 FT-IR spectrometer, respectively. The HRMS were recorded on TMS-AX 505 W instrument. NADPH, cytochrome *c*, reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB) and ethoxyresorufin (EROD) were obtained from Sigma Chemical Company, St. Louis, MO, USA.

2.2. Synthesis and characterization of polyphenolic acetates

Syntheses of 7,8-diacetoxy-4-methylcoumarin (**1**) and 7-acetoxy-4-methylcoumarin (**2**) were described in our earlier communication [9]. The compounds **2** and **7** were obtained from the collection of the (Late) Professor T. R. Seshadri, FRS, former Head, Department of Chemistry, University of Delhi, India. The compounds **4** [10], **5** [11], **6** [12], **14** [13], **15** [14], **11** [15], **10** [16] and **16** and **17** [17] were synthesized according to literature procedures.

2.2.1. 3-3′-Methylene-bis(4,7,8-triacetoxycoumarin) (*9*)

Formaldehyde (77 mg, 2.5 mmol) was added to a hot solution of 4,7,8-trihydroxycoumarin [18] (1 g, 5.1 mmol) in ethanol (10 ml) and the reaction mixture was refluxed. The reaction was monitored by TLC; on completion (1 h), the reaction mixture was cooled and the product obtained was separated by filtration, and crystallized from alcohol to yield white needles of 3,3′-methylene-bis(4,7,8-trihydroxycoumarin) [19]. This hexahydroxycoumarin (300 mg) was dissolved in pyridine (10 ml) and acetic anhydride (1 ml) was added. The reaction mixture was stirred at 40 °C for 6 h. The progress of the reaction was monitored by TLC; on completion, the reaction mixture was poured onto ice-cold water. The solid that precipitated out was filtered and washed with petroleum ether, dried and recrystallized from $CHCl_3$ to afford **9** in 90% yield as a white solid, m.p. 220–222 °C; IR (Nujol): 2926, 1782, 1733, 1678, 1620, 1459, 1371, 1204, 1169, 1079, 1032, 888 and 579 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.32 (18H, bs, 6 × $COCH_3$), 3.80 (2H, s, CH_2), 7.15–7.23 (4H, 2d, J = 9.0 Hz each, 2H each, C-5H and C-5′H and C-6H and C-6′H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 20.66, 20.97, 21.02 and 21.17 (6 × $COCH_3$), 67.25 (CH_2), 115.45 and 117.45 (C-5 and C-5′ and C-6 and C-6′), 119.52 and 120.30 (C-3 and C-3′ and C-10 and C-10′), 130.78 (C-4 and C-4′), 145.78 and 146.02 (C-9 and C-9′ and C-8 and C-8′), 156.26 (C-7 and C-7′), 161.52 (C-2 and C-2′), 167.03, 167.41, 167.86 and 168.84 (6 × $COCH_3$). FAB-HRMS: *m/z* 675.0999 $[M + Na]^+$, $C_{31}H_{24}O_{16}$ + Na. Calcd. 675.0962.

2.2.2. 7,8-Diacetoxy-4-(7,7-dimethyl-1-phenylsulphonyl-3 E-octen-5-ynyl)-2H-1-benzopyran-2-one (*8*)

It was synthesized in three steps [20] from 7,8-dihydroxy-4-methylcoumarin [9]. It was obtained as a white solid, m.p. 104–105 °C; IR (KBr): 3077, 2966, 2927, 2856, 1788, 1739, 1653, 1508, 1447, 1384, 1309, 1273, 1198, 1166, 1149, 1082, 1056, 961, 870, 723 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.17 (9H, s, $C(CH_3)_3$), 2.32 (3H, s, $OCOCH_3$), 2.39 (3H, s, $OCOCH_3$), 2.80 and 3.21 (2H, m, C-2′H), 4.61 (1H, dd, J = 3.7 and 10.8 Hz, C-1′H), 5.57 (1H, d, J = 15.7 Hz, C-4′H), 5.66 (1H, dt, J = 7.0 and 15.7 Hz, C-3′H), 6.33 (1H, s, C-3H), 7.03 (1H, d, J = 9.0 Hz, C-6H), 7.42–7.56 (4H, m, C-5H and C-3″H, C-4″H and C-5″H) and 7.73 (2H, d, J = 7.4 Hz, C-2″H and C-6″H); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 20.57 ($OCOCH_3$), 20.97 ($OCOCH_3$), 30.05 (C-7′), 31.14 and 31.23 [$C(CH_3)_3$], 32.02 (C-2′), 63.85 (C-1′), 76.65 (C-6′), 100.41 (C-5′), 115.93 (C-3′), 117.37 (C-3), 117.72 (C-10), 118.94 (C-6), 121.38 (C-5), 129.71 and 129.77 (C-2″, C-3″, C-5″ and C-6″), 133.80 (C-4″), 135.09 (C-1″), 135.21 (C-4), 136.09 (C-4′), 145.79 (C-8), 145.96 (C-9), 146.44 (C-7), 158.38 (C-2), 167.50 ($OCOCH_3$), 167.79 ($OCOCH_3$). HRMS: *m/z* 537.1597 $[M + H]^+$, $C_{29}H_{28}O_8S$ + H. Calcd. 537.1583.

2.2.3. 7-Acetoxy-4-(7,7-dimethyl-1-phenylsulphonyl-3 E-octen-5-ynyl)-2H-1-benzopyran-2-one (*12*)

It was synthesized in two steps [20] from 7-hydroxy-4-bromomethylcoumarin [20]. It was obtained as a white solid, m.p. 176–177 °C; IR (KBr): 2925, 2363, 1757, 1735, 1618, 1390, 1311, 1269, 1215, 1144, 1081, 970, 927 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.16 (9H, s, $C(CH_3)_3$), 2.34 (3H, s, $-OCOCH_3$), 2.77 and 3.22 (2H, m, C-2′H), 4.64 (1H, dd, J = 3.8 and 10.8 Hz, C-1′H), 5.55 (1H, d, J = 15.8 Hz, C-4′H), 5.67 (1H, dt, J = 6.8 and 15.8 Hz, C-3′H), 6.30 (1H, s, C-3H), 6.98 (dd, 1H, J = 2.0 and 8.7 Hz, C-6H), 7.10 (1H, d, J = 2.0 Hz, C-8H), 7.42 (1H, d, J = 8.7 Hz, C-5H), 7.46–7.62 (3H, m, C-3″H, C-4″H and C-5''H), 7.75 (2H, d, J = 7.3 Hz, C-2″H and C-6″H); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 21.42 ($OCOCH_3$), 29.68 (C-7′), 32.22 (C-2′), 31.13 [$C(CH_3)_3$], 63.65 (C-1′), 76.65 (C-5′), 100.26 (C-6′), 111.17 (C-8), 115.77 (C-4′), 116.74 (C-10), 117.01 (C-6), 118.52 (C-3), 125.19

(C-5), 129.66 and 129.78 (C-2″, C-3″, C-5″, C-6″), 133.86 (C-4″), 135.11 (C-3′), 136.19 (C-1″), 146.30 (C-4), 153.74 (C-7), 154.52 (C-9), 159.62 (C-2) and 168.78 ($OCOCH_3$). HRMS: *m/z* 479.1487 $[M+H]^+$, $C_{27}H_{26}O_6S + H$. Calcd. 479.1528.

2.2.4. 7-Acetoxy-4-(1-phenylsulphonylheptyl)-2H-1-benzopyran-2-one (*13*)

It was synthesized in three steps [19] from 7-hydroxy-4-bromomethylcoumarin [21]. It was obtained as a white solid, m.p. 137–138 °C; IR (KBr): 3084, 2952, 2922, 2856, 1762, 1725, 1618, 1560, 1447, 1385, 1309, 1271, 1216, 1145, 1132, 1082, 1015, 923, 867, 829, 727 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 0.83 (3H, t, $J = 6.3$ Hz, C-7′H), 1.20–1.25 (6H, br s, C-4′H, C-5′H and C-6′H), 2.02 (2H, m, C-3′H), 2.34 (3H, s, $OCOCH_3$), 2.39 (2H, m, C-2′H), 4.57 (1H, dd, $J = 2.5$ and 8.4 Hz, C-1′H), 6.34 (1H, s, C-3H), 6.96 (1H, dd, $J = 2.0$ and 7.3 Hz, C-6H), 7.09 (1H, d, $J = 2.0$ Hz, C-8H), 7.42–7.58 (4H, m, C-5H, C-3″H, C-4″H and C-5″H) and 7.76 (2H, d, $J = 7.5$ Hz, C-2″H and C-6″H); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 14.22 (C-7′), 21.43 ($OCOCH_3$), 22.74–30.05 (C-3′, C-4′, C-5′ and C-6′), 31.60 (C-2′), 64.45 (C-1′), 111.19 (C-8), 116.48 (C-10), 116.95 (C-6), 118.72 (C-3), 124.96 (C-5), 129.54 and 129.76, (C-2″, C-3″, C-5″ and C-6″) 134.86 (C-4″), 138.43 (C-1″), 147.33 (C-4), 149.20 (C-7), 153.72 (C-9), 159.78 (C-2), 168.77 ($OCOCH_3$). EI-HRMS: *m/z* 442.1421 $[M]^+$, $C_{24}H_{26}O_6S$, Calcd. 442.1450.

3. Results

The relative specificities of the acetoxy derivatives of various classes of heterocyclic polyphenolic compounds (Fig. 1) to the liver microsomal TAase have been studied. The results in Fig. 2 demonstrated the TAase catalyzed inhibition of

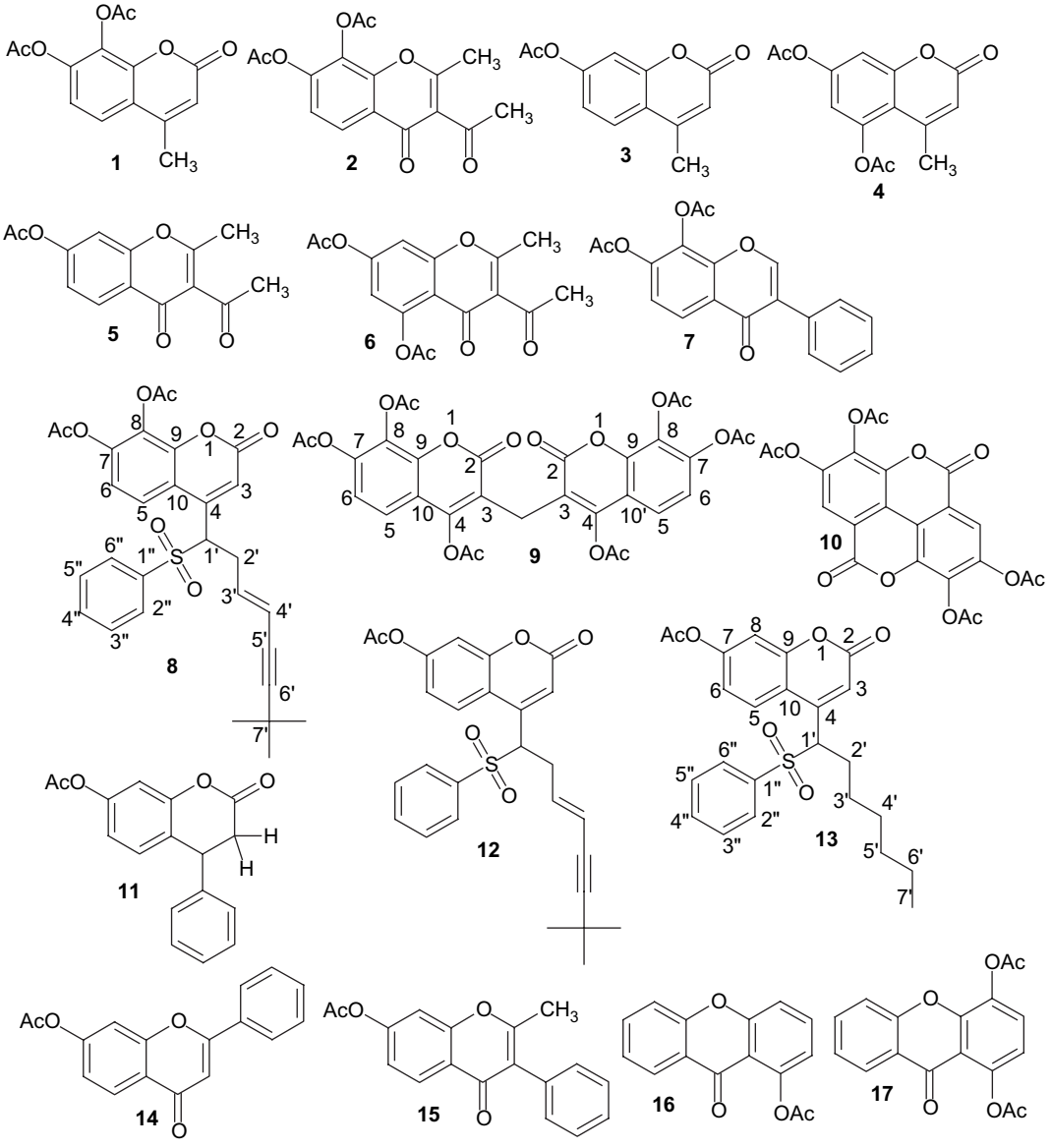

Fig. 1. The structures of polyphenolic acetates.

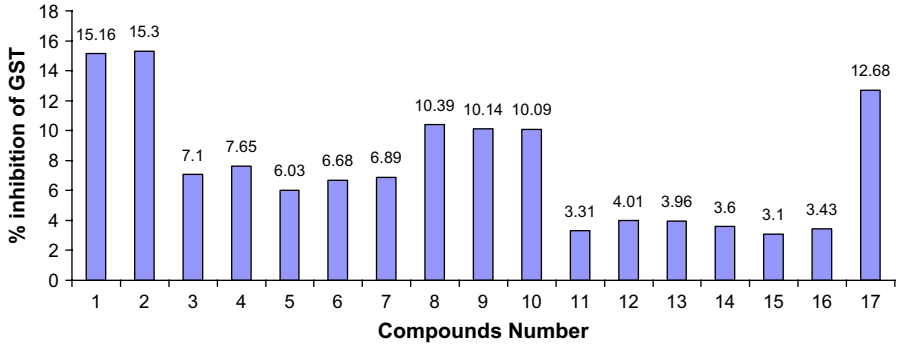

Fig. 2. Comparison of relative specificities of various acetyl polyphenols to liver microsomal acetoxy drug: protein transacetylase.

- TAase activity (preincubation/10 min) expressed in terms of % inhibition of GST under condition of the assay and indicated on top of the bar.
- Concentration of test compounds was 50 μM.
- Values are mean of four observations with variations <5%.

cytosolic GST by polyphenolic acetates, 7,8-diacetoxy-3-acetyl-2-methylchromone (DAAMC, **2**) caused a significant inhibition of TAase that is quite identical to that of the model acetoxy drug **1**. The extent of inhibition of GST by acetoxychromones **5** and **6** is quite identical to that of the monoacetoxycoumarin **3** and diacetoxycoumarin **4**. The compounds **7** and **8** having phenyl substituent at different positions on the pyran ring showed reduction in TAase activity as compared to diacetoxycoumarin **1** and diacetoxychromone **2**. Similar trend also persisted for the compounds **11**–**15** when compared to monoacetoxycoumarin **3**, diacetoxycoumarin **4**, monoacetoxychromone **5** and diacetoxychromone **6**. Surprisingly the biscoumarin **9** bearing three acetoxy groups at the C-4, C-7 and C-8 positions exhibited less TAase activity when compared to **1** and **2**, and the tetra-acetyl ellagic acid **10**, which is the bis counterpart of **1**, yielded significantly lower TAase activity when compared to **1**, however, the TAase activities of compounds **9** and **10** are comparable (Fig. 2). Similarly, reduction in TAase activity was observed for monoacetoxycoumarins **11**, **12** and **13** (as compared to that of **3**) having a bulky substituent at C-4 position of the coumarin skeleton (compounds **12** and **13**); interestingly like the 100% increase in activity of diacetoxycoumarin **1** and diacetoxychromone **2** over that of its monoacetoxy analogue **2**, the corresponding diacetoxycoumarin **8** is twice active as its monoacetoxycoumarin analogues **11**, **12** and **13** (Fig. 2). The TAase activity of monoacetoxyxanthone **16** was found to be significantly less than that of the monoacetoxycoumarin **3**, diacetoxycoumarin **4**, monoacetoxychromone **5** and the diacetoxychromone **6**. This further supports our hypothesis that the phenyl substituent on pyran ring decreases the TAase activity of these benzopyranone compounds. The polyphenolic acetates were also examined for their ability to irreversibly activate liver microsomal NADPH cytochrome *c* reductase, which is another protein substrate for the action of TAase (Fig. 3). It is interesting to note that various concentrations of polyphenolic acetates cause the activation of the reductase (catalyzed by TAase) in tune with their specificities of TAase measured in terms of inhibition of cytosolic GST. The TAase-mediated inhibition of liver microsomal cytochrome P-450 linked mixed function oxidase (MFO) by polyphenolic acetates is shown in Fig. 4. Test compounds were preincubated with liver microsomes, followed by the addition of ethoxyresorufin and NADPH in order to assay the activity of ethoxyresorufin-*O* deethylase (EROD), the results of which are shown in Fig. 4. These results conclusively prove the inference drawn earlier on the specificity of these

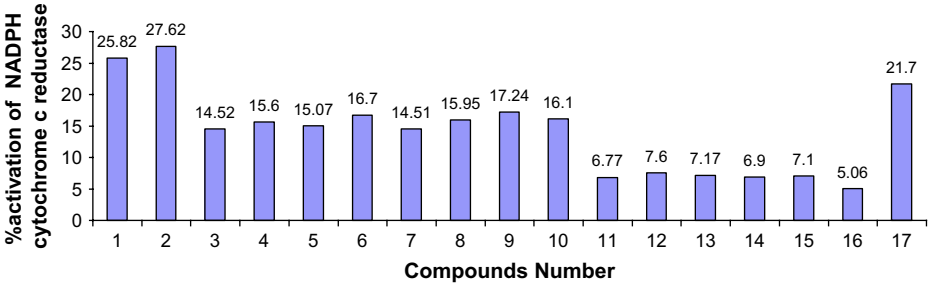

Fig. 3. Activation of liver microsomal NADPH cytochrome *c* reductase by various polyphenolic acetates catalyzed by microsomal acetoxy drug: protein transacetylase.

- TAase activity expressed in terms of % activation/5 min of preincubation of NADPH cytochrome *c* reductase under condition of the assay and indicated on top of the bar.
- Concentration of test compounds was 5 μM.
- Values are mean of four observations with variations <2%.

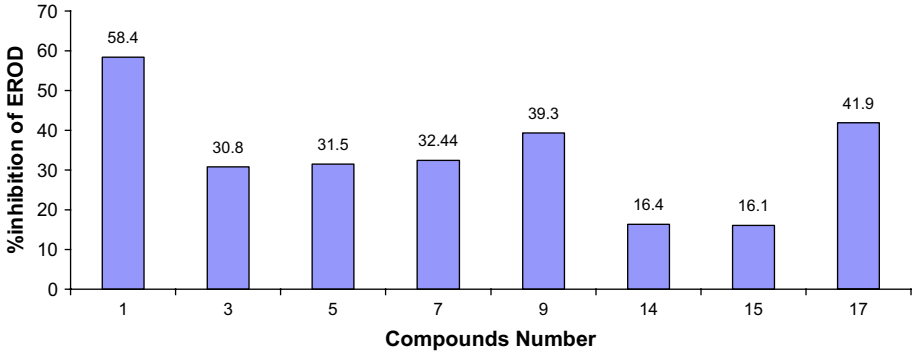

Fig. 4. Inhibition of liver microsomal cytochrome P-450 linked EROD by various polyphenolic acetates catalyzed by acetoxy drug: protein transacetylase.

- The catalytic activity (10 min of preincubation) of cytochrome P-450 linked MFO expressed % inhibition of EROD under condition of the assay and indicated on top of the bar.
- Concentration of the test compound was 25 μM.
- Values are mean of three observations with variations <5%.

compounds to TAase. Accordingly, biscoumarin **9** inhibited cytochrome P-450 linked EROD activity to a lesser extent as compared to diacetoxycoumarin **1**. Similarly, biscoumarin **9** and tetra-acetate of ellagic acid **10** were found to have much less activity to inhibit benzene-induced micronuclei formation in bone marrow cells compared to **1** (Table 1).

The PM3 optimized structures (Fig. 5) suggested that the electron charge distribution and bond order about the active O–Ac bond remain nearly the same in all the systems studied here, the size/flexibility of the substituent around the pyran ring in the substrate seems to control the TAase activity. The longest axis measured (9.756/8.373 Å) for the PM3 optimized geometry of 7,8-diacetoxy-4-methylcoumarin (**1**) (Fig. 5) is found to be much smaller than that of 7-acetoxy-3-acetyl-2-methylchromone (**5**) (11.713/9.673 Å) and that of 1,4-diacetoxyxanthone (**17**) (11.593/10.114 Å) (Fig. 5). This is in agreement with the observed results (Fig. 2) that 7,8-diacetoxy-4-methylcoumarin (**1**) shows greater TAase catalytic activity at a much lower concentration as compared to **5** and **17**. This is because of possible hindrance to the acetoxy group from accessing the active site of TAase resulting in the decreased rate of transfer of the acetyl group to the enzyme. Though 3,3′-methylene-bis[4,7,8-triacetoxycoumarin] (**9**) is expected to show activity comparable to 7,8-diacetoxy-4-methylcoumarin (**1**) due to the presence of 7,8-diacetoxycoumarin moieties in both the compounds, but **9** is only 60% as active as **1** (Fig. 2). The lower activity observed in the case of **9** may be due to lesser proximity of the C-8 acetoxy function to the hetero oxygen (Fig. 5) as compared to that of the C-8 acetoxy function of compound **1** (Fig. 5) due to the steric hindrance arising from the non-planar bulky group present at the C-3 position in the biscoumarin **9** (Fig. 5).

Table 1
Modulation of micronuclei induction in benzene-treated bone marrow cells of rats by acetylated polyphenols

Group[a]	Treatment	Mean	Micronuclei/1000 cells, 95% confidence limit
1	Control	1.25	0.6289–1.8711
2	Benzene	7.60	5.8894–8.7106
3	Benzene + **1**[b]	2.66(65)[c]	1.8101–3.5232
1	Control	1.50	0.4243–2.5757
2	Benzene	7.83	6.8016–8.5650
3	Benzene + **3**[b]	4.33(44.6)[c]	3.4765–5.1919
1	Control	1.75	1.1289–2.3711
2	Benzene	7.50	6.0531–8.9464
3	Benzene + **9**[b]	4.66(37.86)[c]	3.8099–5.5235
1	Control	1.75	1.1199–2.3612
2	Benzene	8.00	6.5160–9.4840
3	Benzene + **10**[b]	4.83(39.62)[c]	3.6066–6.060

Group 1. Control Group: Animals received DMSO 0.1 ml i.p. After 1 h, again DMSO 0.1 ml was given i.p.
Group 2. Benzene Group: DMSO 0.1 ml was given i.p. After 1 h, benzene 0.03 ml/kg BW in 0.1 ml DMSO was given i.p.
Group 3. Benzene + Test compound: Test compound 300 mg/kg BW in 0.1 ml DMSO was given i.p. After 1 h, was again test compound 300 mg/kg BW in 0.1 ml DMSO along with benzene 0.03 ml/kg BW 0.1 ml DMSO given i.p.
[a] Four male rats weighing approximately 100 gm were taken in each case. All animals were sacrificed after 26 h and bone marrow cells were harvested and stained micronuclei were scored as described earlier [8].
[b] These numbers denote the test compound number (Fig. 1).
[c] Numbers in parenthesis denote the percentage of inhibition of formation of micronuclei in benzene-treated bone marrow cells due to test compound.

4. Discussion

The earlier work carried out in our laboratory clearly established the role of the novel enzyme termed as acetoxy drug: protein transacetylase (TAase) catalyzing the transfer of acetyl group from the polyphenolic acetates to receptor proteins. Accordingly, the convincing biochemical evidences were furnished to demonstrate the possible acetylation of enzyme proteins: liver microsomal P-450 dependent mixed function oxidases (MFO), NADPH cytochrome *c* reductase and GST resulting in modulation of their catalytic activities [3,4]. Buffalo liver TAase catalyzed acetylation of GST by **1** was demonstrated, revealing the acetylation of *N*-proline and seven-lysine residues of GST 3-3 [2]. The task of delineating the structural features of the polyphenolic acetates was

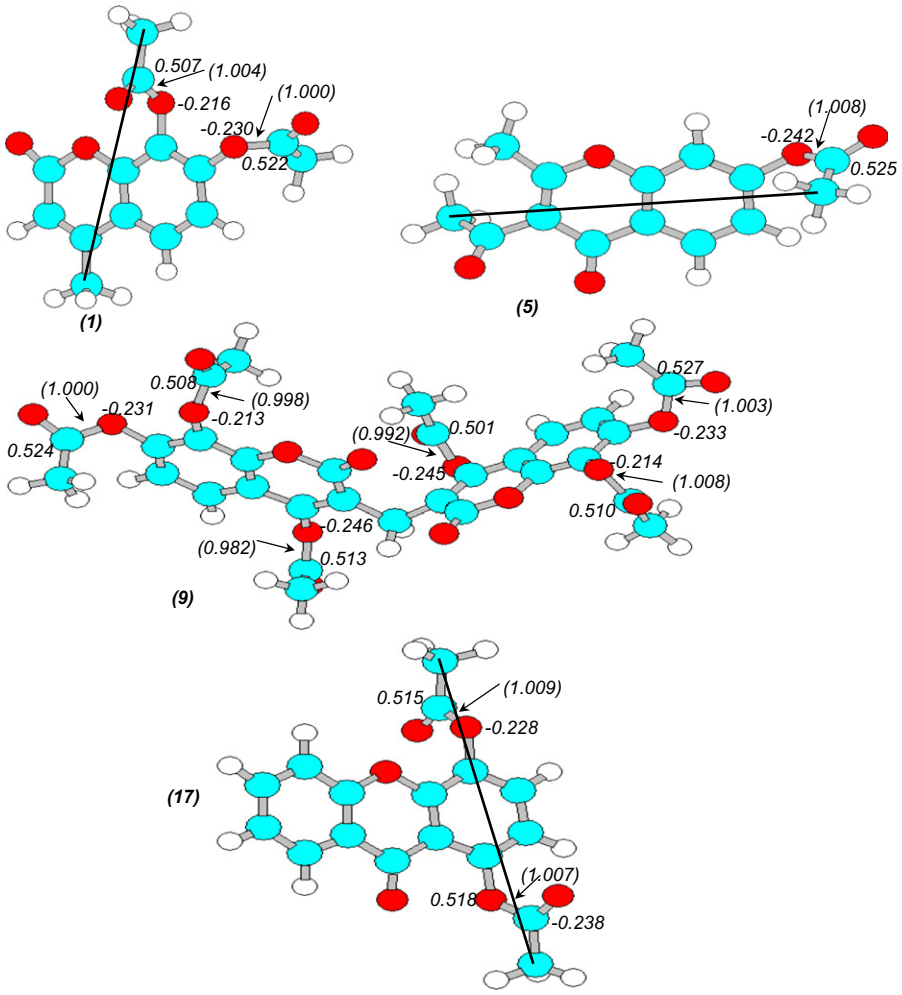

Fig. 5. Optimized molecular structures of 7,8-diacetoxy-4-methylcoumarin (**1**), 7-acetoxy-3-acetyl-2-methylchromone (**5**), 3,3′-methylene-bis[4,7,8-triacetoxycoumarin] (**9**), 1,4-diacetoxyxanthone (**17**) along with charges and bond orders (in parentheses) for selected atoms as obtained by PM3 calculations.

followed by the determination of their specificities to TAase [7]. These investigations examined the positional specificity of acetoxy group to TAase with respect to the oxygen heteroatom of the pyran ring of the polyphenolic acetates. The acetoxy group on the benzenoid ring of the polyphenolic acetates in proximity to the oxygen heteroatom exhibited highest specificity to TAase. Accordingly, 7-acetoxy and 7,8-diacetoxy derivatives of coumarin and flavone/isoflavone yielded the highest activity while the acetoxy group on the pyran ring failed to take part in the TAase catalyzed reaction [5]. The cardinal role of the pyran carbonyl group for the TAase-mediated transfer of acetyl group to the protein substrate was confirmed by the observation that the benzopyran acetates exhibited negligible activity when used as the substrates for TAase [7]. The present investigation laid emphasis on consolidating the influence of

(a) The positions of pyran carbonyl group with respect to the oxygen heteroatom of the polyphenolic acetates.
(b) The substitution by phenyl moiety in pyran nucleus and the effect thereof on the rate of the catalytic activity of liver microsomal TAase.
(c) The specificity of acetoxychromones to TAase as compared to that of the acetoxycoumarins.

It is evident from Fig. 2 that acetoxychromone **2** has similar catalytic activity as compound **1** and similar trend also persisted for the compounds **5** and **6** have similar catalytic activity as the coumarin **3** and **4**. These results clearly indicate that the position of the pyran carbonyl group with respect to the oxygen heteroatom has no effect on the TAase catalyzed transfer of acetyl group to proteins. It was also observed that the pair of compounds **3** and **4** and **5** and **6** have similar catalytic activity but less than compounds **1** and **2**, which strongly indicate that enzyme TAase is more effective in transfer of acetyl groups present in ortho position to each other as compared to meta positions. These facts also clearly indicate that acetyl group at C-5 does not contribute to the effect of acetyl group at C-7 in increasing the rate of transfer of acetyl group to functional proteins as shown in the case of compounds **1** and **2**. We have already confirmed that 5-acetoxy derivative was very poor substrate for TAase [7]. We have also observed that the presence of the phenyl moiety in the pyran nucleus, either as a substituent (as in flavones **7**, **14** and **15**)

or in the fused form (as in acetoxy xanthones **16** and **17**), results in a drastic reduction in their ability to transfer the acetyl group from these compounds to proteins (Figs. 2 and 3). Similarly, acetoxy derivatives of biscoumarin **9** and the compound with condensed coumarins system such as ellagic acid tetraacetate (**10**) exhibit reduced activity when used as substrates for TAase compared to **1** and **2**. The results of the present study have conclusively established that the presence of phenyl group on pyran ring renders their acetoxy derivatives less active as substrates for the liver microsomal TAase. We have earlier demonstrated that the TAase catalyzed acetylation of functional proteins leads to the expression of altered physiological effects, such as the inhibition of clastogenic effects due to the inhibition of microsomal cytochrome P-450 linked mixed function oxidase (MFO)-mediated activation of chemical mutagen like benzene [8]. The inferences of the present investigation revealed that the benzene-induced formation of micronuclei in bone marrow cells is inhibited due to inhibition of P-450 linked benzene epoxidation by acetoxy polyphenolic acetates and the extent of inhibition was proportional to their specificity to TAase. These observations reflect to the conclusion drawn on the structure–activity relations discussed here.

The transfer of acetyl groups from the polyphenolic peracetates to receptor proteins catalyzed by the novel enzyme, acetoxy drug: protein TAase has been also established by our earlier studies [2]. 7,8-Diacetoxy-4-methylcoumarin (**1**), a model acetylated polyphenol was found to modulate the activities of enzyme proteins, such as cytochrome P-450-linked MFO, NADPH cytochrome *c* reductase and cytosolic GST catalyzed by TAase. Our previous studies highlighted the structural features of polyphenolic acetates determining the specificity for liver microsomal TAase [7]. The present study focused attention upon elucidating the SAR drawing on the influence of the presence of phenyl ring on the benzopyran nucleus. For this purpose, the specificities of the acetylated coumarins, biscoumarins, chromones, flavones/isoflavones and xanthones for TAase were compared. The results of these studies demonstrated that the presence of phenyl ring on the pyran nucleus of the polyphenolic acetates drastically reduces their specificity to the TAase. The specificities of various polyphenolic acetates were in the order: $\mathbf{1} = \mathbf{2} > \mathbf{3} = \mathbf{4} = \mathbf{5} = \mathbf{6} = \mathbf{7} < \mathbf{8} = \mathbf{9} = \mathbf{10} > \mathbf{11} = \mathbf{12} = \mathbf{13} = \mathbf{14} = \mathbf{15} = \mathbf{16}$ and $\mathbf{17} < \mathbf{1}$,**2**.

5. Conclusion

A large number of polyphenolic acetates were synthesized and characterized and structures were confirmed using spectral data. They were examined for their abilities to modulate TAase-mediated action such as inhibition of glutathione *S*-transferase, cytochrome P-450 linked MFO and activation of NADPH cytochrome *c* reductase and related biological effects in order to establish the specificities of TAase to various classes of PA with special reference to the effect of phenyl moiety/bulky group on benzopyran nucleus.

6. Experimental protocols

6.1. Animals

Male albino rats of Wistar strain weighing around 180–200 g fed on rat chow were used.

6.2. Preparation of liver microsomes and cytosol

Rats were sacrificed by decapitation, liver was removed and a 30% homogenate was prepared in 10 mM phosphate buffer containing 0.25 M sucrose and 1.4 mM β-mercaptoethanol and pH adjusted to 7.0. The homogenate was centrifuged at $10{,}000 \times g$ for 30 min in a Sorvall superspeed centrifuge and the supernatant was spun at $100{,}000 \times g$ in a Beckman ultracentrifuge (Model L-7) at 4 °C. The cytosolic fraction was set aside at −20 °C. The microsomal pellet was resedimented and suspended in 0.25 M sucrose. Protein contents of microsomes and cytosol were assayed by the method of Lowry et al. [22].

6.3. Assay of acetoxy drug: protein transacetylase (TAase)

TAase was assayed using the coumarin **1** and cytosolic GST as the substrates as per the details given in our earlier reports [3]. The assay mixture consisted of 0.25 M phosphate buffer (pH 6.5), liver microsomes (25 μg protein), polyphenolic acetate (added in 50 μl DMSO), liver cytosol (10–15 μg protein) and water to make up a volume of 0.5 mL. The contents of the tube were scaled up as per requirement and preincubated at 37 °C for various periods of time. The aliquots were removed periodically into a spectrophotometer cuvette containing GSH and CDNB to make up their concentration 1 mM in a total volume of 1 mL and the progress of the GST activity was followed at 340 nm using a Cary spectrophotometer (Cary Bio 100). In control samples, polyphenolic acetates were replaced by DMSO. The unit of TAase was expressed in terms of % inhibition of GST under the conditions of the assay and ensured that the reaction was linear with respect to enzyme concentration and incubation time.

6.4. TAase-mediated biochemical actions of polyphenolic acetates

6.4.1. Modulation of NADPH cytochrome c reductase

The method consisted of preincubation of test compounds (polyphenolic acetates) with microsomes, followed by addition of substrates for the reductase assay (cytochrome *c* and NADPH) as described earlier [4]. The rat liver microsomes (40 μg protein) were mixed with test compound, 0.05 M phosphate buffer (pH 7.7) and water to make 0.5 mL volume. 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 a spectrophotometer cuvette (1 cm light path) containing 0.1 mM EDTA, 36 mM cytochrome *c* and 1 mM NADPH in a total volume of 1 mL.

The progress of the reaction was followed by monitoring absorption at 550 nm. In the control samples, test compounds were replaced by DMSO. The increment in the reductase activity due to polyphenolic peracetates over the control was expressed as % activation.

6.4.2. Modulation of ethoxyresorufin deethylase (EROD)

Assay of EROD was carried out by the method of Lubet et al. [23]. The reaction mixture consisted of 0.05 M Tris–HCl buffer (pH 7.5), 25 mM $MgCl_2$, 10 μM ethoxyresorufin and 1 mg of rat liver microsomal protein. The reaction was initiated by the addition of 125 μM NADPH and the rate of dealkylation of resorufin was followed by measuring the fluorescence using excitation $\lambda = 522$ nm and emission $\lambda = 586$ nm. For the modulation, liver microsomes were preincubated with test compound as described earlier. The reaction mixture for the assay of EROD was added at the end of preincubation and the rate of dealkylation of resorufin was followed as described above.

6.4.3. Modulation of benzene-induced micronuclei formation in rat bone marrow cells

6.4.3.1. Treatment. Male albino rats of Wistar strain (150–200g) were administrated separately, the test compound (300 mg/kg/b wt dissolved in 0.1 ml DMSO) intraperitoneally (i.p.) and intratracheally (it), followed by a second dose of the test compound along with a dose of benzene (3 mg/kg b wt in DMSO). A group of rats were injected with benzene alone, while control animals received DMSO alone. The animals were sacrificed 26 h after the last injection.

6.4.3.2. Isolation of bone marrow and lung cells, preparation of smear and staining. Bone marrow cells were isolated by flushing femora or rats with 2 mL of Hank's Balanced Salt Solution (HBSS). For preparation of smear and staining, the method of Schmid was followed as adapted by Raj et al. [8].

*6.4.3.3. Optimization of structures of 7,8-diacetoxy-4-methylcoumarin (**1**), 7-acetoxy-3-acetyl-2-methylchromone (**5**), 3,3′-methylene-bis(4,7,8-triacetoxycoumarin) (**9**) and 1,4-diacetoxyxanthone (**17**)*. A semiempirical quantum chemical method was used to study the structures of various coumarins, chromones and xanthone. The molecular spatial structures, atomic charges, and bond order analysis of these systems were theoretically estimated using HyperChem Release 5.1 Pro [24] Quantum Chemistry Package. The initial geometries of the systems 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 [25,26] Hamiltonian through the eigenvector following (EF) routine [27] on restricted Hartree–Fock basis without any conformational or symmetry restrictions. As one cannot deny the possibility of the involvement of water molecules in protein–ligand binding which is not resolved in crystal structures, macroscopic solvent effects were investigated within the framework of continuum solvation model using the COSMO (Conductor like Screening Model) [28] method as implemented in MOPAC 97 in the CS MOPAC Pro Version 5.0 with dielectric constant for water taken as 78.5 at 298 K. This is a good approximation for solvents of high relative permittivity as reported by us earlier [29].

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