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# Mechanism of Biochemical Action of Substituted 4-Methylbenzopyran-2-ones. Part 9: Comparison of Acetoxy 4-Methylcoumarins and Other Polyphenolic Acetates Reveal the Specificity to Acetoxy Drug: Protein Transacetylase for Pyran Carbonyl Group in Proximity to the Oxygen Heteroatom

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**Abstract**—The evidences for the possible enzymatic transfer of acetyl groups (catalyzed by a transacetylase localized in microsomes) from an acetylated compound (acetoxy-4-methylcoumarins) to enzyme proteins leading to profound modulation of their catalytic activities was cited in our earlier publications in this series. The investigations on the specificity for transacetylase (TA) with respect to the number and positions of acetoxy groups on the benzenoid ring of coumarin molecule revealed that acetoxy groups in proximity to the oxygen heteroatom (at C-7 and C-8 positions) demonstrate a high degree of specificity to TA. These studies were extended to the action of TA on acetates of other polyphenols, such as flavonoids and catechin with a view to establish the importance of pyran carbonyl group for the catalytic activity. The absolute requirement of the carbonyl group in the pyran ring of the substrate for TA to function was established by the observation that TA activity was hardly discernible when catechin pentacetate and 7-acetoxy-3,4-dihydro-2,2-dimethylbenzopyran (both lacking pyran ring carbonyl group) were used as the substrates. Further, the TA activity with flavonoid acetates was remarkably lower than that with acetoxycoumarins, thus suggesting the specificity for pyran carbonyl group in proximity to the oxygen heteroatom. The biochemical properties of flavonoid acetates, such as irreversible activation of NADPH cytochrome C reductase and microsome-catalyzed aflatoxin $B_1$ binding to DNA in vitro were found to be in tune with their specificity to TA.


## Introduction

In our previous communications, evidences were documented for the existence of an enzyme (transacetylase) localized in rat liver microsomes catalyzing the transfer of acetyl group from the model compounds, namely acetoxy 4-methylcoumarins to specific enzyme proteins, such as cytochrome P-450, NADPH cytochrome-C-reductase and glutathione-*S*-transferase (GST). A simple procedure was developed for the assay of liver microsomal transacetylase (TA) based on the irreversible inhibition of GST (protein substrate) with the use of a model compound 7,8-diacetoxy-4-methylcoumarin (DAMC, **17**, the acetyl group donor) as the second substrate. The extent of inhibition of GST was considered proportional to the DAMC–protein transacetylase activity.[1] 7,8-Dihydroxy-4-methylcoumarin (DHMC), one of the products of DAMC–protein transacetylase reaction was identified although the demonstration of protein acetylation per se is still being persued in our laboratories. In a preliminary study, we have found that apart from

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DAMC, several other acetylated polyphenolic compounds, such as acetoxyflavonoids are substrates for the TA.[2] Hence DAMC–protein transacetylase could be termed in general as 'acetoxy drug–protein transacetylase'. In this communication, we have focused our attention on the seminal role of the pyran ring carbonyl group in determining the specificity for acetoxy drug: protein transacetylase using acetylated derivatives of flavonoids, coumarins and benzopyrans. The results of these studies have revealed the specificity of the pyran ring carbonyl group in proximity to the pyran ring oxygen hetero-atom for efficient transfer of acetyl groups to proteins by the TA.

## Results

The relative specificities of the acetates of polyphenolic compounds such as flavonoids, coumarins and catechin to liver microsomal TA have been compared with a view to assess the importance of pyran ring carbonyl group in influencing the catalytic activity of the enzyme. For this purpose, phenolic acetates (PAs, the test compounds) were pre-incubated with the liver microsomes in the presence of rat liver cytosol for different periods of time, followed by addition of CDNB and GSH, in order to assay GST. The inhibition of GST under the conditions of assay was considered to be proportional

Table 1. Specificity to acetoxy drug: protein transacetylase for acetylated polyphenolic substrates

| Test compound no. | Concentration (μM) | Time of pre-incubation (min) | Transacetylase (units) | Test compound no. | Concentration (μM) | Time of pre-incubation (min) | Transacetylase (units) |
|---|---|---|---|---|---|---|---|
| **1** | 50 | 10 | 3.10 | **12** | 50 | 10 | 7.70 |
| | | 20 | 6.00 | | | 20 | 15.5 |
| | | 30 | 8.80 | | | 30 | 22.8 |
| | | 40 | 11.20 | | | 40 | 29.1 |
| **2** | 50 | 10 | 3.90 | **13** | 50 | 10 | 7.81 |
| | | 20 | 7.90 | | | 20 | 14.12 |
| | | 30 | 11.40 | | | 30 | 19.23 |
| | | 40 | 19.60 | **14** | 50 | 10 | 6.89 |
| **3** | 50 | 10 | 3.60 | | | 20 | 11.52 |
| | | 20 | 7.00 | | | 30 | 16.92 |
| | | 30 | 10.3 | | | 40 | 27.23 |
| | | 40 | 14.0 | **15** | 50 | 10 | 6.50 |
| **4** | 50 | 10 | 4.50 | | | 20 | 11.90 |
| | | 20 | 7.90 | | | 30 | 17.40 |
| | | 30 | 11.4 | | | 40 | 24.60 |
| | | 40 | 19.6 | **16** | 100 | 10 | 0.07 |
| **5** | 50 | 10 | 0.90 | | | 20 | 1.40 |
| | | 20 | 1.90 | | | 30 | 1.90 |
| | | 30 | 2.50 | | | 40 | 2.10 |
| | | 40 | 3.60 | **17** | 25 | 10 | 8.20 |
| **6** | 50 | 10 | 0.60 | | | 20 | 15.9 |
| | | 20 | 1.20 | | | 30 | 24.4 |
| | | 30 | 1.60 | | | 40 | 30.2 |
| | | 40 | 2.30 | **18** | 50 | 10 | 7.70 |
| **7** | 50 | 10 | 0.60 | | | 20 | 15.0 |
| | | 20 | 1.20 | | | 30 | 21.10 |
| | | 30 | 1.60 | | | 40 | 26.40 |
| | | 40 | 2.30 | **19** | 200 | 10 | 5.20 |
| **8** | 50 | 10 | 0.95 | | | 20 | 9.60 |
| | | 20 | 1.90 | | | 30 | 16.70 |
| | | 30 | 2.80 | | | 40 | 21.60 |
| | | 40 | 3.90 | **20** | 50 | 10 | 0.04 |
| **9** | 50 | 10 | 4.90 | | | 20 | 0.08 |
| | | 20 | 9.70 | | | 30 | 1.10 |
| | | 30 | 15.0 | | | 40 | 1.60 |
| | | 40 | 18.9 | **21** | 200 | 10 | 3.10 |
| **10** | 100 | 10 | 8.60 | | | 20 | 5.90 |
| | | 20 | 16.3 | | | 30 | 8.70 |
| | | 30 | 23.8 | | | 40 | 11.50 |
| | | 40 | 30.4 | | | | |
| **11** | 50 | 10 | 3.40 | | | | |
| | | 20 | 6.60 | | | | |
| | | 30 | 10.0 | | | | |
| | | 40 | 13.1 | | | | |

The details are given under 'Materials and Methods'. The values are the mean of three observations with variation $<2\%$.

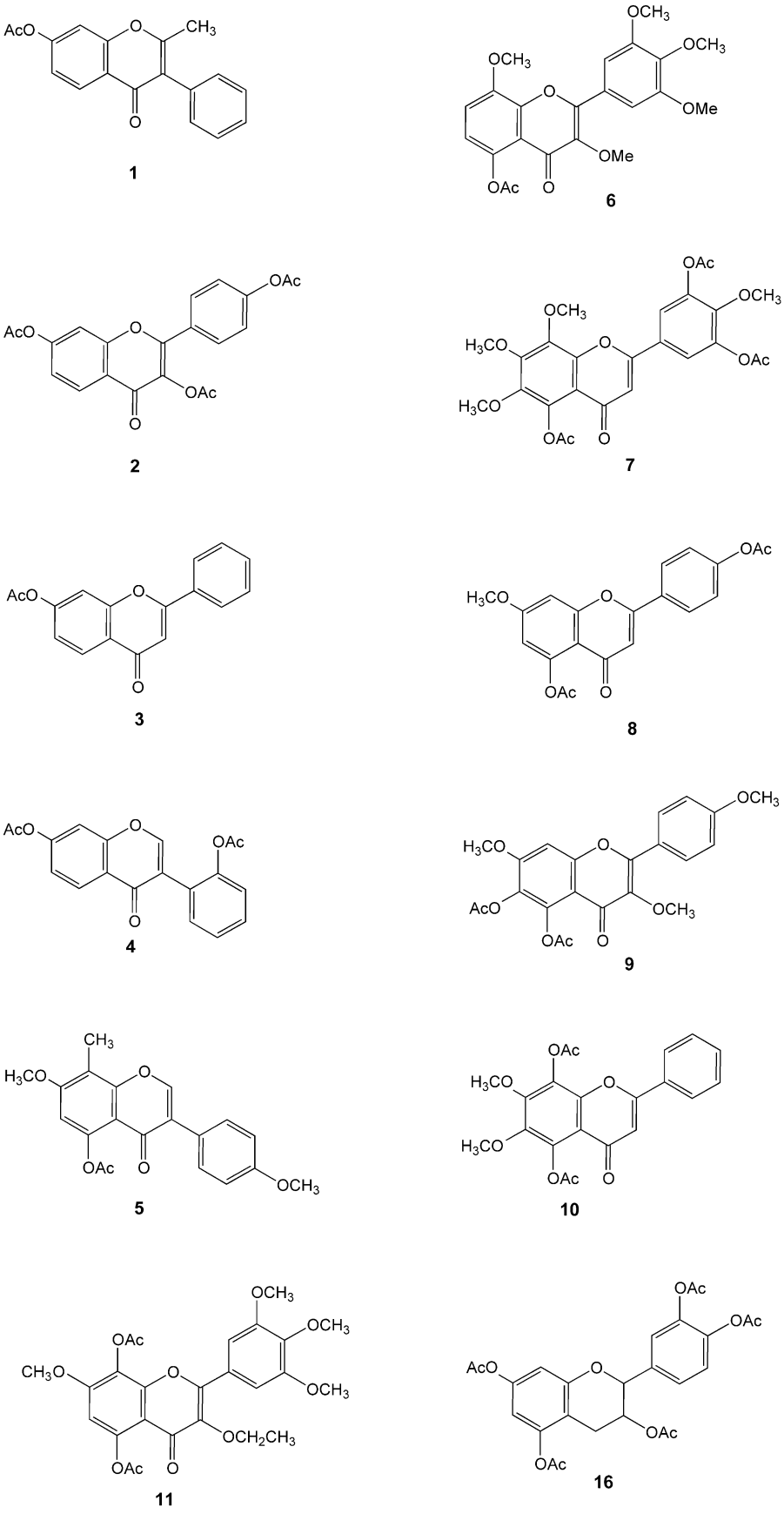


**Figure 1.** Structures of acetylated phenolics.

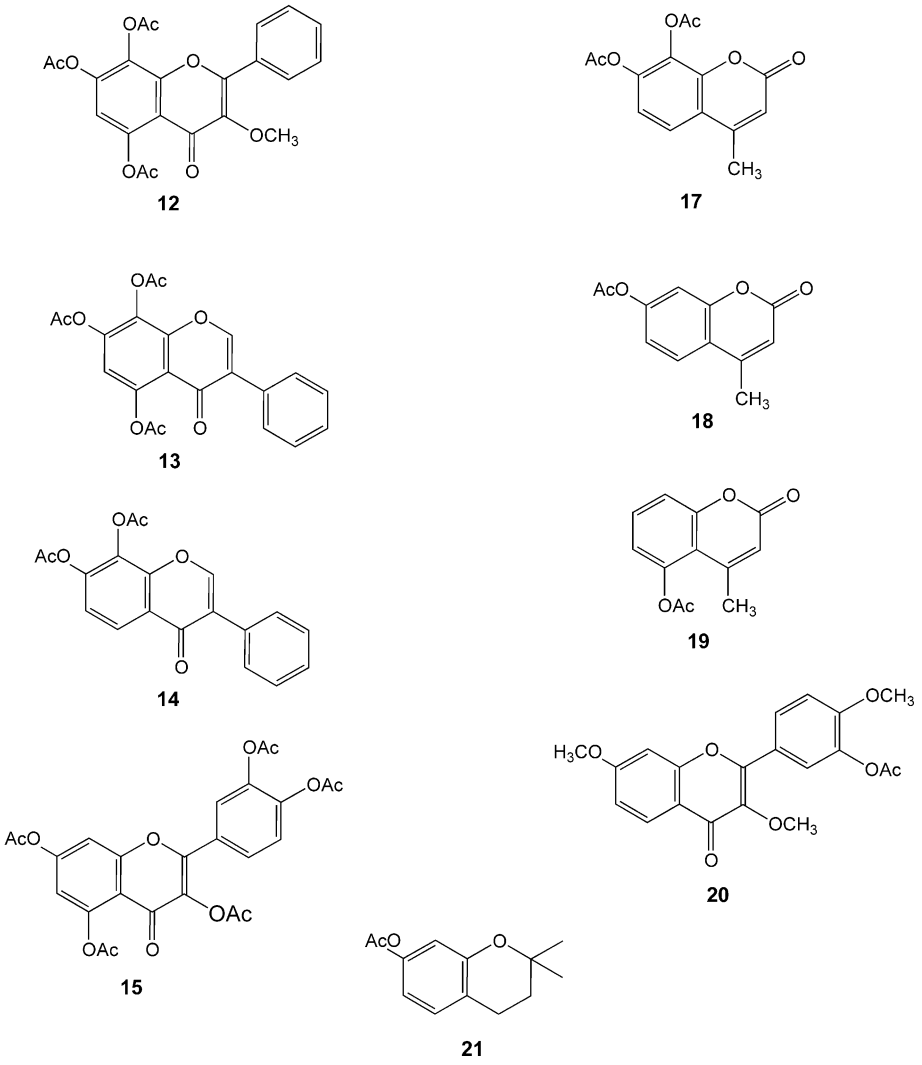


**Figure 1.** (*continued*)

to the TA activity as described in our earlier publications.[1,3,4] The results documented in Table 1 make it abundantly clear that PAs demonstrate varying degrees of specificity to the TA. Among the flavonoid peracetates containing one acetoxy group in the benzenoid ring fused to the pyran ring, the 7-acetoxyisoflavones **1** and **4**, and 7-acetoxyflavones **2** and **3** yielded maximum activity when used as the substrates for TA (Table 1). The 5-acetoxyflavones **6**, **7** and **8**, as well as the 5-acetoxyisoflavone **5** were found to be poor substrates for TA and yielded only around 1/5th the activity compared to those of 7-acetoxyflavones and 7-acetoxyisoflavones, respectively (Table 1). The flavones/isoflavones containing two or three acetoxy groups in the benzenoid ring fused to the pyran ring, that is the compounds **9**–**15** showed higher TA activity as compared to those of 7-monoacetoxy derivatives **1**–**4**. Though the activities of the flavonoid peracetates did not exhibit any rigid, uniform trend, their effects on TA followed the order: 7,8 diacetoxy = 5,7,8 triacetoxy = 7 acetoxy > 5,8 diacetoxy > 5,6 diacetoxy > 5,7 diacetoxy > 5 acetoxy derivatives. This conclusion points out the fact that the acetoxy groups at C-7 and C-8 positions are most ideally suited and make the substrate containing them quite active in facilitating the TA- catalyzed transfer of acetyl groups to the proteins, while the acetoxy groups at other positions on the benzenoid ring (either at C-6 or C-5) hardly contribute to the TA catalytic activity. It is also clear from the results documented here that the acetoxy groups on the side phenyl ring in flavonoids do not contribute to the enzymatic transfer of acetyl groups. The specificity with respect to acetyl group(s) in flavonoids was found to be quite valid when compared to acetoxy 4-methylcoumarins. 7-Acetoxy-4-methylcoumarin (**18**) was found to be a superior substrate in comparison to the 5-acetoxy-4-methylcoumarin (**19**), the best activity was exhibited by 7,8-diacetoxy-4-methylcoumarin (DAMC, **17**). The fact that catechin pentacetate (**16**) and the acetoxybenzopyran **21** (both lacking the pyran ring carbonyl group) proved to be poor substrates for the TA, substantially supports that the pyran ring carbonyl group is required for the enzymatic transfer of acetyl groups from the acetyl donor (PA) to the receptor proteins. NADPH Cytochrome C reductase activation (Table 2) was taken as another yardstick to assess the activity and specificity of these substrates. A comparison of the results demonstrated in Tables 1 and 2 reveals that the compounds with struc-

Table 2. Transacetylase mediated activation of NADPH cytochrome C reductase by various acetylated polyphenols

| Test Compound No. | Concentration (μM) | Time of preincubation (min) | NADPH-cytochrome C reductase (% activation) | Test Compound No. | Concentration (μM) | Time of preincubation (min) | NADPH-cytochrome C reductase (% activation) |
|---|---|---|---|---|---|---|---|
| **1** | 10 | 5 | 6.90 | **12** | 10 | 5 | 18.40 |
| | | 10 | 14.10 | | | 10 | 35.40 |
| | | 20 | 25.30 | | | 20 | 68.0 |
| | | 30 | 32.60 | | | 30 | 91.60 |
| **2** | 10 | 5 | 6.30 | **13** | 5 | 5 | 16.30 |
| | | 10 | 12.20 | | | 10 | 30.78 |
| | | 20 | 22.90 | | | 20 | 52.57 |
| | | 30 | 34.60 | | | 30 | 83.57 |
| **3** | 10 | 5 | 7.10 | **14** | 5 | 5 | 14.51 |
| | | 10 | 13.80 | | | 10 | 26.51 |
| | | 20 | 26.70 | | | 20 | 39.79 |
| | | 30 | 38.80 | | | 30 | 72.60 |
| **4** | 10 | 5 | 9.20 | **15** | 5 | 5 | 7.10 |
| | | 10 | 17.70 | | | 10 | 13.30 |
| | | 20 | 30.50 | | | 20 | 25.0 |
| | | 30 | 46.30 | | | 30 | 37.30 |
| **5** | 10 | 5 | 4.40 | **16** | 5 | 5 | 3.10 |
| | | 10 | 7.90 | | | 10 | 6.00 |
| | | 20 | 15.60 | | | 20 | 11.50 |
| | | 30 | 24.90 | | | 30 | 17.20 |
| **6** | 10 | 5 | 3.90 | **17** | 2 | 5 | 10.20 |
| | | 10 | 7.60 | | | 10 | 18.90 |
| | | 20 | 15.60 | | | 20 | 39.90 |
| | | 30 | 21.80 | | | 30 | 57.60 |
| **7** | 10 | 5 | 2.20 | **18** | 2 | 5 | 5.80 |
| | | 10 | 4.10 | | | 10 | 11.90 |
| | | 20 | 8.50 | | | 20 | 20.40 |
| | | 30 | 12.0 | | | 30 | 35.20 |
| **8** | 10 | 5 | 2.30 | **19** | 5 | 5 | 4.50 |
| | | 10 | 4.50 | | | 10 | 9.250 |
| | | 20 | 8.80 | | | 20 | 16.80 |
| | | 30 | 13.10 | | | 30 | 25.95 |
| **9** | 10 | 5 | 7.70 | **20** | 10 | 5 | 3.60 |
| | | 10 | 15.0 | | | 10 | 7.10 |
| | | 20 | 28.80 | | | 20 | 13.50 |
| | | 30 | 39.80 | | | 30 | 23.80 |
| **10** | 10 | 5 | 8.20 | **21** | 10 | 5 | 3.60 |
| | | 10 | 16.0 | | | 10 | 7.60 |
| | | 20 | 30.30 | | | 20 | 13.40 |
| | | 30 | 44.90 | | | 30 | 19.50 |
| **11** | 10 | 5 | 7.10 | | | | |
| | | 10 | 14.40 | | | | |
| | | 20 | 26.90 | | | | |
| | | 30 | 37.50 | | | | |

The details are given under 'Materials and Methods'. The values are mean of three observations with variation $< 2\%$.

tural features demonstrating high specificities to TA also effect a high degree of irreversible activation of the reductase. Results tabulated in Table 3 reveal the inhibitory potential of PAs on liver microsomes catalyzed $AFB_1$ binding to DNA in vitro. DAMC is found to be a powerful inhibitor of $AFB_1$ epoxidation catalyzed by P-450 (Table 3). The pattern of inhibition of $AFB_1$–DNA binding by acetylated flavonoids is in tune with their specificities to the TA (Tables 1 and 3). Figure 2a–h demonstrate the optimized structures of some of the acetoxyflavonoids studied in this report. We had earlier demonstrated[4] that the C-8 acetoxy group in 7,8-diacetoxy-4-methylcoumarin (**17**) bends more towards the pyran ring oxygen hetero-atom due to the presence of neighboring bulky C-7 acetoxy group, and **17** is a very good substrate for the TA. This observation is also valid in the case of flavonoid acetates **10**, **12**, **13** and **14** (Fig. 2c–f, respectively; Tables 1 and 2).

## Discussion

The earlier work carried out in our laboratory unraveled the existence of a membrane bound enzyme in liver and other tissues catalyzing the transfer of acetyl group of the model compound 7,8-diacetoxy-4-methyl-

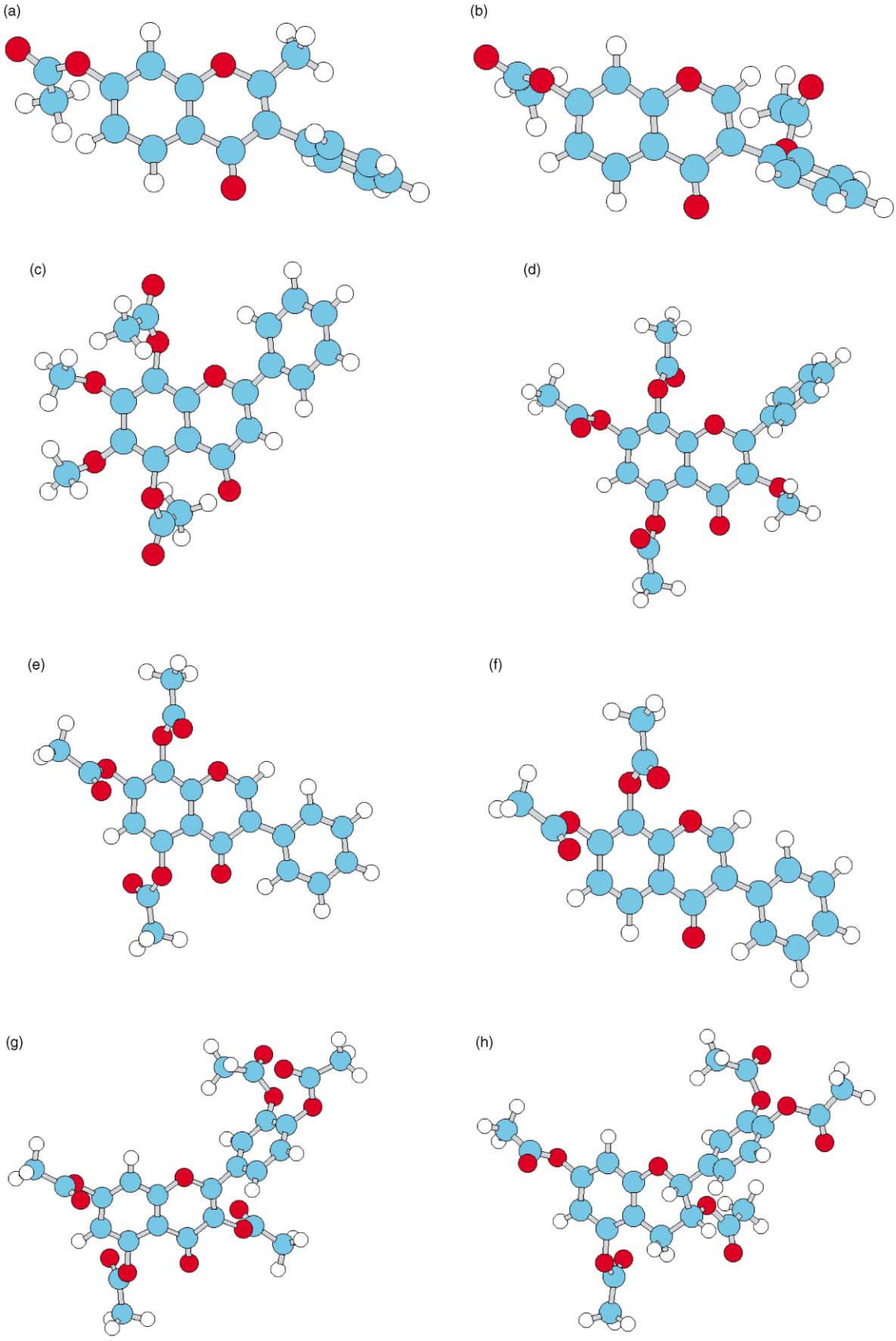


**Figure 2.** Optimized Structure of (a) **1**; (b) **4**; (c) **10**; (d) **12**; (e) **13**; (f) **14**; (g) **15**; (h) **16**.

coumarin (DAMC) to enzyme proteins such as GST, cytochrome P-450 and NADPH cytochrome C reductase resulting in the modulation of their catalytic activities.[1,3,4] The present study has projected acetoxyflavonoids as substrates for the TA reaction. The results from this study clearly demonstrate that acetoxycoumarins yield remarkably higher activity with liver microsomal TA as compared to acetoxyflavonoids (Table 1). The drastic reduction in the affinity for flavonoid acetates to the TA is largely due to the fact that the pyran carbonyl group is not in proximity to the oxygen heteroatom as in the case of coumarins. The cardinal role of the pyran carbonyl group of polyphenolic substrates to the catalytic activity of TA is quite evident from the fact that catechin pentacetate (**16**), which lacks a carbonyl group is a very poor substrate for TA unlike quercetin pentaacetate (**15**), which is similar to the former in structure (Table 1). This conclusion is substantiated by the fact that the 7-acetoxybenzopyran **21** (also lacking the pyran carbonyl group) contributed to TA catalyzed transfer of acetyl group to almost 50% extent than the corresponding 7-acetoxycoumarin **18** (Tables 1–3). It is noteworthy that only the acetoxy groups on the benzenoid ring of benzopyranone nucleus of flavones are acted upon by the microsomal TA, while the acetoxy groups on the side-chain phenyl ring (as in the corresponding counterpart compounds **2**, **4**, **7**, **8** and **20**) as well as the acetoxy groups on the pyranone ring of the flavone (as in **2**) hardly contribute to the catalytic activity. The TA mediated biochemical action of polyphenol PAs, that is, the activation of NADPH cytochrome C reductase (Table 2) and the inhibition of $AFB_1$ binding to DNA (Table 3) also agree with these results. These studies have also revealed the fact that the TA activity is largely independent of the influence of the side-chain groups (other than the acetates) on its catalytic activity.

Similar to the case of our earlier studied corresponding acetoxycoumarins,[4] the optimized structures of PAs (Fig. 2a–h) have also revealed the orientation of the C-8 acetoxy group in the C-7,C-8 diacetoxy flavones and isoflavones being more towards the pyran ring oxygen heteroatom. The pyran ring of catechin pentaacetate (**16**) was found distorted (Fig. 2h), the lack of double bond between C-2 and C-3 results in the loss of conjugation. Further, the carbons at C-3 and C-4 in catechin pentaacetate (**16**) are $sp^3$ hybridized resulting in the induction of strain in the dihydrobenzopyran ring, which could be another reason for its ineffectiveness as a substrate for TA. We have earlier shown by carrying out the X-ray diffraction studies[5] on the 6-hydroxy analogue of the 7-acetoxy-3,4-dihydrobenzopyran **21** (which also is a poor substrate for the TA) that there is indeed a strain in the dihydrobenzopyran ring in such compounds. Thus, this structural feature, in addition to the absence of the carbonyl group in compounds **16** and **21** also contributes to their ineffectiveness as substrates for TA.

**Table 3.** Effects of polyphenolic peracetates on liver microsome-mediated $AFB_1$-DNA binding inhibition in vitro

| Test compound no. | $AFB_1$–DNA binding (p-mole $AFB_1$ bound/mg DNA/30 min) | % Inhibition |
|---|---|---|
| Control | 203.5 | — |
| **2** | 186.5 | 9.6 |
| **5** | 198.0 | 2.9 |
| **9** | 190.5 | 6.6 |
| **12** | 178.5 | 12.5 |
| **17** | 85.1 | 58.2 |
| **18** | 128.7 | 37.7 |
| **19** | 166.1 | 18.4 |
| **21** | 198.1 | 2.9 |

The details are given under 'Materials and Methods'. The values are mean of three observations with variation <2%.

The di-/triacetoxyflavonoids **12**, **13** and **14** (Fig. 2d–f, respectively) showed high affinity towards TA (Tables 1 and 2) as all the three compounds carry the C-7,C-8-diacetoxy substitution pattern and the C-7 acetoxy group in each case pushes the C-8 acetoxy function close to the pyran ring oxygen hetero-atom. Similarly the two methoxy groups at the C-6,C-7 positions in the C-5,C-8-diacetoxyflavone **10** push the C-8 acetoxy function nearer the pyran ring oxygen hetero-atom (Fig. 2c), thus making it a good substrate for the TA (Tables 1 and 2). In conclusion, best TA activity is exhibited by the benzopyranones carrying either two acetoxy groups at the C-7/C-8 positions or those carrying at least one acetoxy group at the C-8 position, but having fully substituted benzenoid ring. Synthetic and activity evaluation studies on thio-carbonyl analogues of these acetoxybenzopyranones towards TA are being presently carried out in our Laboratories and the results shall be communicated in a future publication.

## Materials and Methods

### Chemicals

NADPH, cytochrome C, reduced glutathione (GSH), aflatoxin $B_1$ ($AFB_1$), 1-chloro-2,4-dinitrobenzene (CDNB), quercetin, (+)-catechin and calf thymus DNA were purchased from Sigma Chemical Company, St. Louis, MO (USA). [$^3$H] $AFB_1$-(G) was obtained from Moravek Biochemicals, Brea, CA (USA). All other laboratory reagents were of high grade and obtained from local suppliers.

### Animals

Male albino rats of wistar strain weighing around 180–200 g, fed on rat chow supplied by Hindustan Lever Ltd., Mumbai (India) were used.

### Synthesis and characterization of various polyphenolic acetates (Fig. 1)

Compounds **1**–**5**, **7**–**10** and **20** were synthesized and characterized according to the literature procedures mentioned in references,[6–16] respectively. The compounds **6** and **11**–**14** were obtained form the collection of the (Late) Professor T. R. Seshadri, FRS, Depart-

ment of Chemistry, University of Delhi. Compounds **17** and **18** were synthesized and characterized according to our earlier report.[17,18] Compound **19** was synthesized and characterized according to the procedure of Sen and Bagchi.[19] Quercetin and (+)-catechin were acetylated using acetic anhydride/pyridine/DMAP method to give the compounds **15** and **16**, respectively, which were fully identified from their physical and spectral data. 7-Acetoxy-3,4-dihydro-2,2-dimethylbenzopyran (**21**) was prepared by the method of Levai and Timar.[20]

### Preparation of microsomes and cytosol

Rats were killed by decapitation, liver removed and 30% homogenate (w/v) was prepared in 10 mM phosphate buffer containing 0.25 M sucrose and 1.4 mM β-mercaptoethanol, pH adjusted to 7.0. The homogenate was centrifuged at 10,000*g* for 30 min and the supernatant was spun at 100,000*g* for 1 h in the Beckman Ultracentrifuge Model L7. The cytosolic fraction was set aside at −20 °C. The microsomal pellet was resuspended in 1.15% KCl, microsomes were resedimented and suspended in 0.25 M sucrose. Protein content of microsomes and cytosol were assayed by the method of Lowry et al.[21]

### Assay of acetoxy drug: protein transacetylase

The principle governing the TA assay and the detailed procedure were elaborated by us in our earlier report.[1] The assay was carried out using polyphenolic peracetates (PAs) as the first substrate and cytosolic GST as the second substrate. The assay mixture in a total volume of 0.8 mL consisted of 0.25 M phosphate buffer (pH 6.5), liver microsomes (25.0 μg protein), PA dissolved in DMSO (100 μM), liver cytosol (12.5–15.0 μg protein) and water to make up to a total volume of 0.8 mL. The contents of the tube (scaled up as per requirement) were preincubated at 37 °C. The aliquots (0.8 mL portion) were removed periodically into a spectrophotometer cuvette containing CDNB and GSH to make their final concentration of 1 mM in a total volume of 1.0 mL and GST activity was assayed by following absorption at 340 nm.[22] The units of TA present (Table 1) were expressed in terms of percent inhibition of GST under the conditions of the assay.

### Transacetylase mediated biochemical action of polyphenolic peracetates

**Modulation of NADPH Cytochrome C-reductase.** The method consisted of preincubation of PA with microsomes, followed by addition of substrates for the reductase assay (Cytochrome C and NADPH) as described earlier.[3] The rat liver microsomes (40 μg protein) were mixed with PA (5 μM), 0.05 M phosphate buffer (pH 7.7), and water to make 0.5 mL volume. The contents (scaled up as per requirement) were pre-incubated at 37 °C in a shaking water bath. The aliquots (0.5 mL portion) were removed periodically into 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, PA were replaced by DMSO. The increment in reductase activity due to PA over the control was expressed as percent activation.

Liver microsome catalysed $AFB_1$ binding to DNA in vitro and inhibition by PA was carried out as described in our earlier communication.[18]

### Analysis of optimized structures of various polyphenolic peracetates

Geometries of the compounds **1**, **4**, **10**, **12**, **13**, **14**, **15** and **16** were optimized using PM3 method[23] as implemented in HyperChem Release 5.1 Pro Quantum Chemistry Package.[24] The preliminary optimized structures were subjected to a systematic conformational search and the lowest energy conformation was optimized without geometric constraints using RHF/ PM3, followed by vibrational frequency analysis. For the solvent effect, rigorous calculations can be performed using Gaussian 94[25] but no significant deviation in the predicted structural trends is expected since the effect of solvent can be considered to be similar for all molecules having same functional groups.

## Acknowledgements

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