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# Mechanism of Biochemical Action of Substituted 4-Methylbenzopyran-2-ones. Part 10: Identification of Inhibitors for the Liver Microsomal Acetoxycoumarin: Protein Transacetylase

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**Abstract**—The quantitative structure–activity relationship (QSAR) studies conducted by us earlier revealed the cardinal role of the pyran ring carbonyl group in the acetoxy polyphenolic compounds for the acetoxy polyphenol:protein transacetylase (TAase) activity. Hence, an attempt was made to examine whether such substrate analogues of benzopyran acetates which lack in the pyran ring carbonyl group, such as 7-acetoxy-2,3-dihydro-2,2-dimethylbenzopyran (BPA), cetachin pentaacetate (CPA) and hematoxylin pentaacetate (HPA) could inhibit the 7,8-diacetoxy-4-methylcoumarin (DAMC):protein (glutathione-*S*-transferase) transacetylase activity. These compounds were indeed found to remarkably inhibit the TAase activity in a concentration dependent manner and exerted their inhibitory action very rapidly. Further BPA, CPA and HPA were found to abolish the TAase mediated activation of NADPH cytochrome C reductase as well as the inhibition of liver microsome catalyzed aflatoxin $B_1$ ($AFB_1$)-DNA binding by DAMC very effectively. These results strongly suggest that the acetoxybenzopyrans merit as potent inhibitors of TAase.


## Introduction

Our earlier investigations in this series[1–3] conclusively evidenced the presence of a unique enzyme in liver and other tissues possibly catalyzing the transfer of acetyl groups from a model acetoxycoumarin (DAMC) to certain receptor proteins, such as glutathione *S*-transferase (GST), cytochrome P-450-linked mixed function oxidases and NADPH cytochrome C reductase resulting in the modulation of their catalytic activities. The enzyme was termed as DAMC: Protein Transacetylase (TAase) which was assayed[3] using GST as the second substrate, the inhibition of GST under the conditions of the assay represented TAase activity.[3] TAase was purified from mammalian liver microsomes to a reasonable degree of purity, nevertheless need was felt to devise an affinity chromatographic procedure for the purification of TAase. In order to accomplish this task, we undertook QSAR studies[4] which gave the hint for the possible inhibitors of TAase. We have in this communication identified several benzopyran acetates as inhibitors of TAase.

## Materials and Methods

### Chemicals

NADPH, cytochrome C, reduced glutathione (GSH), $AFB_1$, 1-chloro-2,4-dinitrobenzene (CDNB), (+)-catechin, hematoxylin and calf thymus DNA were purchased

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from Sigma Chemical Company, St. Louis, MO, USA. [$^3$H] $AFB_1$ was the product of Moravek Biochemicals, Brea, CA, USA.

### Synthesis of 7,8-diacetoxy-4-methylcoumarin (DAMC) and inhibitors of TAase (Fig. 1)

(a) 7,8-Dihydroxy-4-methylcoumarin (DHMC) was synthesized by the well-known Pechmann condensation of pyrogallol with ethyl acetoacetate, its diacetyl derivative (DAMC) was prepared by acetylation of DHMC as described by us earlier.[5]
(b) 7-Acetoxy-3,4-dihydro-2,2-dimethylbenzopyran (BPA) was synthesized according to the procedure of Levai and Timar.[6]
(c) Catechin pentaacetate (CPA) and hematoxylin pentaacetate (HPA) were prepared by the acetylation of (+)-catechin and hematoxylin, respectively by acetic anhydride/pyridine/DMAP method and were fully characterized from their $^1$H and $^{13}$C NMR spectral data and comparison with the data in literature.[4,7]

### Animals

Male albino rats of Wistar strain weighing around 150–200 g fed on rat chow supplied by Hindustan Lever Ltd., Mumbai (India) were used.

### 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*g* for 30 min in a Sorvall superspeed centrifuge and the supernatant was spun at 100,000*g* in a Beckman ultracentrifuge (Model L-7). The cytosolic fraction was set aside at −20 °C. The microsomal pellet was resedimented and suspended in 0.25 M sucrose. Protein content of microsomes and cytosol were assayed by the method of Lowry et al.[8]

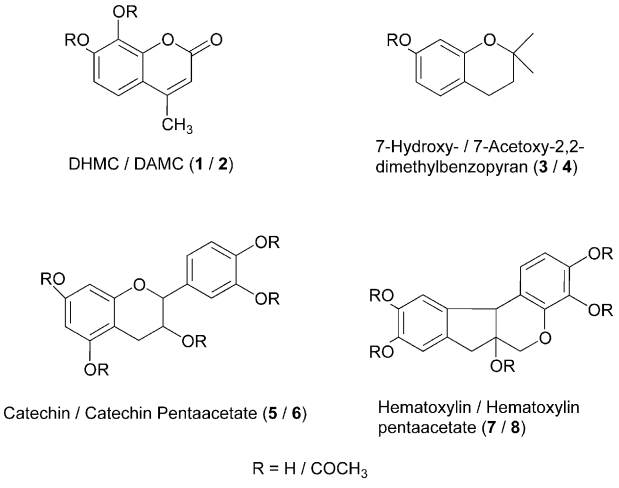


**Figure 1.** Structures of the substrate and the inhibitors of TAase.

### Assay of TAase

TAase was assayed using DAMC 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), DAMC (100 μM) added in 50 μL DMSO, liver cytosol (10–15 μg protein) and water to make up 0.5 mL. The contents of the tube were scaled up as per requirement and preincubated at 37 °C for various periods. The aliquots were removed periodically into a spectrophotometer cuvette containing CDNB and GSH 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 Bio100).[9] In control samples, DAMC was 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.

### Inhibition of TAase activity

(a) The inhibitors BPA, CPA and HPA were separately preincubated with microsomes and cytosolic GST, followed by the assay of GST as described above in order to quantify the activity of TAase (Table 1).
(b) The inhibitors CPA, BPA and HPA were separately included in the reaction mixture for TAase as described above at a concentration of 100 μM and preincubated along with DAMC (25 μM), liver microsomes (25 μg protein) and liver cytosol (15 μg protein) for various periods, followed by the assay of GST in order to quantify the TAase activity. Unit of TAase activity was plotted against the time of incubation (Fig. 2).
(c) The inhibitors CPA, BPA and HPA were separately included in the reaction mixture of TAase at concentrations varying from 10 to 150 μM and preincubated for 10 min along with DAMC

**Table 1.** Specificity of BPA, CPA and HPA for acetoxy drug protein: transacetylase

| Time of preincubation[a] (min) | TAase units[b] | | | |
|---|---|---|---|---|
| | DAMC (25 μM) | BPA (100 μM) | CPA (100 μM) | HPA (100 μM) |
| 10 | 8.20 | 5.50 | 6.40 | 3.90 |
| 20 | 15.80 | 6.30 | 9.60 | 5.30 |
| 30 | 24.40 | 7.80 | 10.90 | 7.00 |
| 40 | 30.20 | 8.40 | 12.70 | 8.10 |

[a]BPA, CPA and HPA were preincubated with liver microsomes, followed by the assay of GST as described under 'Materials and Methods'.
[b]TAase unit is expressed in terms of percent inhibition of GST under the assay conditions. The values are the average of five separate observations with error <5%.

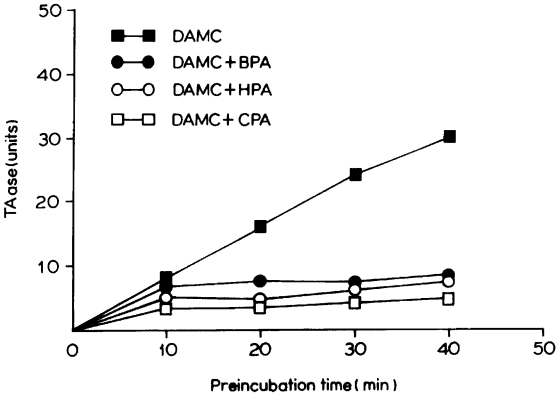


**Figure 2.** Inhibition of TAase by BPA,CPA and HPA. The detailed assay procedure is described under 'Materials and Methods'. TAase activity was expressed in terms of percent inhibition of GST under the conditions of the assay. The values are the average of three values with error $<5\%$.

(100 μM), liver microsomes (25 μg), liver cytosol (15 μg protein) and TAase was assayed as described above. The reaction mixture containing DAMC devoid of inhibitor served as control. The inhibitory action was expressed by plotting TAase activity as percentage of control against the concentration of the inhibitor (Fig. 3).

### Inhibition of transacetylase mediated biochemical actions

**Inhibition of modulation of NADPH cytochrome C reductase by DAMC catalyzed by TAase.** The method consisted of preincubation of DAMC with the inhibitor and the liver microsomes, followed by the addition of substrates for the reductase (cytochrome C and NADPH) for the assay as described earlier.[2] The rat liver microsomes (40 μg protein) were preincubated with DAMC (5 μM), 0.05 M phosphate buffer (pH 7.0), the inhibitor (100 μM) in DMSO and water to make 0.5 mL volume. The contents, scaled up as per requirement were preincubated at 37 °C for varying periods. The aliquot (0.5 mL) was 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.[10] In the appropriate blank sample, DAMC/inhibitor was replaced by DMSO. The percentage activation of reductase was plotted against time of preincubation (Fig. 4).

**Effect of TAase inhibition on the liver microsomes mediated $AFB_1$-DNA binding by DAMC (Table 3).** Liver microsomes (1 mg protein) were pre-incubated separately with 100 μM phosphate buffer (pH 7.4), 100 μM inhibitor (CPA/BPA/HPA) for 30 min at 37 °C followed by the addition of [$^3$H] $AFB_1$ (250 μCi/μM), 2 mM NADPH, and 0.1 mg calf thymus DNA in a total volume of 1 mL and the incubation was continued for another 30 min. After incubation, 2 mL of extraction mixture (phenol/chloroform/isoamyl alcohol, 50:50:1 v/v) was added and DNA was isolated by the procedure of Wang and Cerutti,[11] as described in detail in our earlier publication.[12] In the appropriate blank sample, DAMC/inhibitor was replaced by DMSO.

## Results and Discussion

A novel enzyme termed as acetoxy drug: protein transacetylase (TAase) localized in liver microsomes carrying out the possible transfer of acetyl group of acetylated xenobiotics such as acetoxy substituted 4-methylcoumarins, flavones, isoflavones, xanthones and chalcones to specific enzyme proteins leading to modulation of their catalytic activities has been characterized in our previous publications.[1–4,13,14] DAMC was used as a model substrate for the assay of TAase as described above. TAase was partially purified and characterized from mammalian liver and human placenta.[3,4,15] The QSAR study of various acetylated polyphenols, that is acetoxycoumarins, acetoxyflavones and acetoxyisoflavones

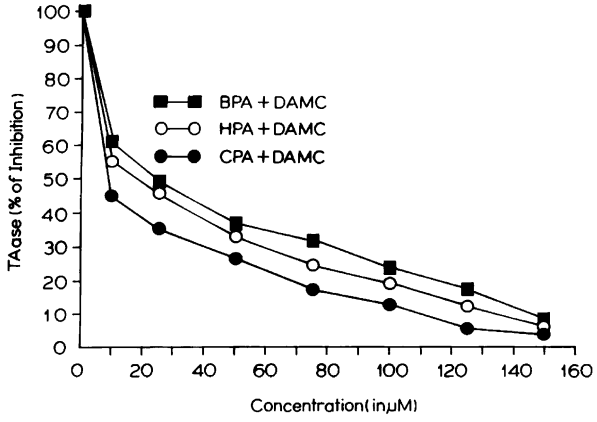


**Figure 3.** Effect of the concentration of the inhibitors on the activity of TAase. The inhibitors BPA/CPA/HPA at the concentration of 10–150 μM were separately included in the reaction mixture of TAase as described under 'Materials and Methods'. The values are the average of three separate observations with error $<5\%$.

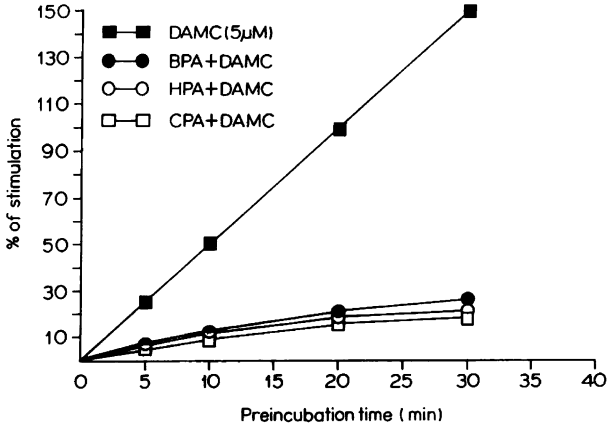


**Figure 4.** Effect of the inhibitors on the liver microsomal TAase catalyzed activation of NADPH cytochrome C reductase by DAMC. The detailed assay procedure is described under 'Materials and Methods'. The values are the average of three separate observation with error $<2\%$.

**Table 2.** Inhibitory potential of the DAMC: GST transacetylase inhibitors

| Inhibitor | $IC_{50}$ (μM) |
|---|---|
| BPA | 39.00 |
| CPA | 33.00 |
| HPA | 41.00 |

Assay of the transacetylase activity using DAMC and cytosolic GST as the substrates was carried out as described under 'Materials and Methods'. Dose–response plot of enzyme fractional and activity ($v_i/V_o$) was plotted against inhibitor concentration. The concentration of the inhibitor required to achieve a half maximal degree of inhibition ($IC_{50}$) was determined graphically.[16]

**Table 3.** Reversal of transacetylase mediated inhibition of liver microsome catalysed $AFB_1$–DNA binding by DAMC

| Incubation mixture[a] | $AFB_1$–DNA binding pmoles $AFB_1$ bound/30 min/mg protein |
|---|---|
| DMSO | 209.2 |
| DAMC | 86.2 |
| BPA | 206.7 |
| CPA | 205.5 |
| HPA | 204.8 |
| BPA + DAMC | 207.1 |
| CPA + DAMC | 208.9 |
| HPA + DAMC | 208.3 |

[a]The concentration of DAMC, BPA/CPA/HPA was 100 μM. The values are the average of three separate observations with variation <5%.

revealed the following features specific for TAase: (a) the presence of acetoxy groups in proximity to the oxygen heteroatom, and (b) absolute requirement of the presence of the pyran ring carbonyl group.[4] Accordingly, acetoxy groups at the C-7 and C-8 positions on the benzenoid ring of polyphenols gave highest TAase activity. The acetylated polyphenols, such as BPA, CPA and HPA lacking the pyran ring carbonyl group supported little activity when used as substrates for the TAase[4] (Table 1). It was thought interesting to examine whether the compounds BPA, CPA and HPA which are substrate analogues could act as the inhibitors for TAase. For this purpose, the test compound (BPA/CPA/HPA) was preincubated with liver microsomes and DAMC, followed by GST assay for the TAase activity as described under 'Materials and Methods'. The results shown in Table 1 demonstrate TAase activity when the test compounds were used as substrates. It is clear from the data that BPA/CPA/HPA are very poor substrates for the TAase. An effort was made to examine the outcome of the inclusion of the test compound(s) in the incubation mixture for the assay of TAase activity. The data shown in Figure 2 highlighted the remarkable inhibition of TAase activity by BPA, CPA and as well as HPA. The incubation of BPA/CPA/HPA for 10 min at 100 μM concentration resulted in nearly 75–90% reduction of the TAase activity. Also, it is evident from the data that there is no change in the extent of inhibition at 40 min of preincubation compared to that at 10 min. Hence, these compounds can be considered to exert their inhibitory action rapidly. The inhibitory action of BPA, CPA and HPA was substantiated by the inhibitor concentration-dependent diminution of TAase catalytic activity (Fig. 3). The results shown in Table 2 record considerably close $IC_{50}$ values for TAase inhibition, thus corroborating the aforementioned results (Fig. 2). We have established earlier[2] that DAMC through the action of TAase dramatically activated microsomal NADPH cytochrome C reductase activity. TAase catalyzed activation of NADPH cytochrome C reductase by DAMC was found to be remarkably abolished by inclusion of the inhibitor BPA/CPA/HPA in the preincubation mixture (Fig. 4). The inhibitor(s) alone were not found to have any influence on the activity of the reductase (data not shown). Cytosolic P-450-linked mixed function oxidase proved to be another enzyme protein whose catalytic activity was shown to be effectively inhibited by the TAase mediated action of DAMC.[1] Accordingly, the results included in Table 3 demonstrate that liver microsomal TAase catalyzed inhibition of $AFB_1$ epoxidation (measured as $AFB_1$-DNA binding) by DAMC was very effectively abolished by TAase inhibitors BPA/CPA/HPA, while the inhibitors alone were not found to have any influence on the P-450 catalyzed $AFB_1$-DNA binding.

The aforementioned results clearly established the identification of inhibitors for TAase, the criterion for the TAase inhibitor being the lack of pyran ring carbonyl group in a oxygen containing heterocyclic polyphenolic acetate. There are several motivations for studying the enzyme inhibition. By the study of relative binding affinity of the inhibitors of varying structures, it is possible to get insight into the active site of an enzyme in the absence of a high resolution three-dimensional structure.[14,16] The nature of inhibition of TAase by BPA, CPA and HPA, and their analogues will be described in the ensuing publications.

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