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Mechanism of Biochemical Action of Substituted Benzopyran-2-ones. Part 8: Acetoxycoumarin: Protein Transacetylase Specificity for Aromatic Nuclear Acetoxy Groups in Proximity to the Oxygen Heteroatom

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Abstract—Our earlier work established a convenient assay procedure for acetoxycoumarin (AC): protein transacetylase (TA) by indirectly quantifying the activity of glutathione (GSH)-S-transferase (GST), the extent of inhibition of GST under the conditions of the assay represented TA activity. In this communication, we have probed the specificity for TA with respect to the number and position of acetoxy groups on the benzenoid as well as the pyranone rings of the coumarin system governing the efficient transfer of acetyl groups to the protein(s). For this purpose, coumarins bearing one acetoxy group, separately at C-3 or C-4 position and 4-methylcoumarins bearing single acetoxy group, separately at C-5, C-6 or C-7 position were synthesized and specificities to rat liver microsomal TA were examined. Negligible TA activity was discernible with 3-AC as the substrate, while the substrate efficiency of other AC were in the order 7-acetoxy-4-methylcoumarin (7 AMC) > 6 AMC > 5 AMC = 5 ADMC = 4 AC. To achieve a comparable level of GST inhibition which was proportional to the enzymatic transfer of acetyl groups to the protein (GST), the concentrations of 7-AMC, 6-AMC, 5-AMC and 4-AC were in the order 1:2:4:4, respectively. One diacetoxycoumarin, i.e., 7,8-diacetoxy-4-methylcoumarin (DAMC) was also examined and it was found to elicit maximum level of GST inhibition, nearly twice that observed with 7-AMC. These observations lead to the logical conclusion that a high degree of acetyl group transfer capability is conferred when the acetoxy group on the benzenoid ring of the coumarin system is in closer proximity to the oxygen heteroatom, i.e., when the acetoxy groups are at the C-7 and C-8 positions.

Introduction

Substituted dioxygenated 4-methylcoumarins were reported to inhibit liver microsomal cytochrome P-450 (P-450) linked mixed function oxidase (MFO) catalysed epoxidation of aflatoxin B_1 (AFB_1). Acetoxy 4-methylcoumarins were found to possess remarkable ability to inhibit AFB_1-epoxidation by MFO, hinting at the possible role of acetoxy groups leading to irreversible inhibition of microsomal P-450.[1] 7,8-Diacetoxy-4-methylcoumarin (DAMC), indeed, was found to effect mechanism based inhibition of P-450 activities,[2] such as microsome mediated AFB_1-epoxidation, dealkylation of alkylated resorufin and toxicokinetics of benzene.[3] We had envisaged that the mechanism of action of DAMC possibly involves the role of a microsomal transacetylase (TA) catalyzing the transfer of acetyl groups from DAMC to specific proteins.[2,4] On establishing the action of acetoxy methylcoumarin (AMC) as novel acetylating moiety as described above, an effort was made to establish the specificity for TA with respect to the position of acetoxy groups on the benzenoid and pyranone rings of the coumarin system governing the efficient transfer of acetyl groups to protein(s). The results highlight the enzyme specificity for acetoxy groups nearer to the oxygen heteroatom.

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Materials and Methods

Chemicals

NADPH, cytochrome C, reduced glutathione (GSH), AFB_1, 1-chloro-2,4-dinitrobenzene (CDNB) and calf thymus DNA were purchased from Sigma Chemical Company, St. Louis, MO (USA). [3H]AFB_1-(G) was obtained from Moravek Biochemicals, Brea, CA (USA).

Synthesis and characterization of various acetoxycoumarins

(a) 7,8-Dihydroxy-4-methylcoumarin (DHMC) was prepared by Pechman condensation of pyrogallol with ethyl acetoacetate,[5] its diacetoxy derivative (DAMC) was prepared by the acetylation of DHMC with acetic anhydride/pyridine. The structure of DAMC was confirmed by spectral data reported earlier.[6]
(b) 7-Acetoxy-4-methylcoumarin (7-AMC) was synthesized and characterized according to the earlier report. [6]
(c) 6-Acetoxy-4-methylcoumarin (6-AMC) was synthesized and characterized according to the method of Dixit and Padukone.[7]
(d) 5-Acetoxy-4,7-dimethylcoumarin (5-ADMC) was synthesized according to the procedure of Chakravarty et al.[8]
(e) 5-Acetoxy-4-methylcoumarin (5-AMC), 4-acetoxycoumarin (4-AC) and 3-acetoxycoumarin (3-AC) were synthesized and characterized according to the procedure of Sen and Bagchi.[9]

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.

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 and pH adjusted to 7. The homogenate was centrifuged at $10{,}000\times g$ for 30 min and the supernatant was spun at $100{,}000\times g$ for 1 h in the Beckman Ultracentrifuge Model L7. The cytosolic fraction was set aside at $-20\,^{\circ}C$. 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.[10]

Assay of acetoxycoumarin: protein transacetylase

The principle governing the TA assay and the detailed procedure were elaborated by us in our earlier report.[11] The assay was carried out using AC 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 μg protein), AC (100 μM), liver cytosol (12.5–15 μg protein) and water to make up a total volume of 0.8 mL. The contents of the tube (scaled 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.[12]

The unit of TA was expressed in terms of percent inhibition of GST under the conditions of the assay.

Measurement of kinetic constants for acetoxycoumarin: protein transacetylase

The enzyme was assayed as per the procedure described above. The concentration of one of the substrates (AC) was varied from 10–100 μM keeping the concentration of the other substrate (rat liver cytosol GST) constant (15 μg protein) during the preincubation with rat liver microsomes (25 μg protein) for 10 min, followed by the addition of CDNB and GSH for GST assay as mentioned earlier. The reciprocal of the initial rate of TA expressed in terms of percent inhibition of GST under conditions of the assay was plotted against the reciprocal of the AC concentration to deduce the K_m and ν_{max} values.

Transacetylase mediated biochemical action of acetoxycoumarins

(a) Modulation of NADPH cytochrome C-reductase: The method consisted of preincubation of AC with microsomes, followed by addition of substrates for the reductase assay (cytochrome C and NADPH) as described by us earlier.[4] The rat liver microsomes (40 μg protein) were incubated with AC (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 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, AC were replaced by DMSO. The increment in reductase activity due to AC over the control was expressed as percent activation.
(b) Liver microsome catalysed inhibition of AFB_1 binding to DNA in vitro by AC was carried out as described in our earlier communication.[2]

Analysis of optimized structures of various acetoxy 4-methylcoumarins

Geometries of 3-AC, 7-AMC and DAMC were optimized using PM3 method[13,14] as implemented in HyperChem Release 5.1 Pro (HyperChem Release 5.1, Hypercube Inc., USA, 1997) Quantum Chemistry Package.

Results and Discussion

The evidences for the existence of a novel enzyme in mammalian tissues catalyzing the transfer of acetyl

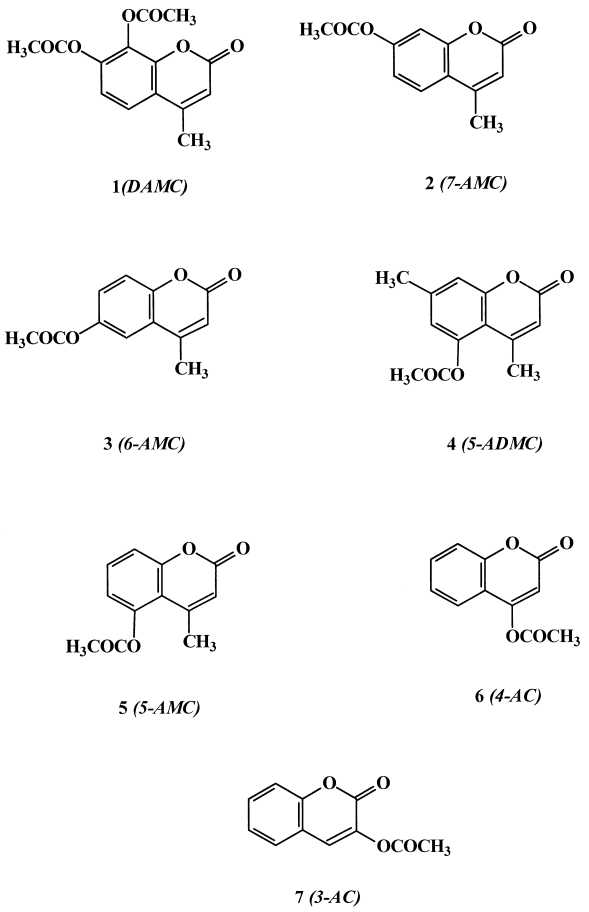

Figure 1. Structures of acetoxycoumarins (AC).

groups from an acetylated compound (acetoxycoumarin) to protein were documented for the first time during the course of our studies on the mode of inhibition of microsomal P-450-linked MFO by the model compound DAMC.[1,2] The facts that (i) P-450-linked MFO inhibition by DAMC did not involve oxidative metabolism of the inhibitor, (ii) the profound inhibition elicited by DAMC was totally abolished by thiol blocking agent *p*-hydroxymercuribenzoate and (iii) DHMC, the deacetylated product of DAMC failed to produce mechanism-based inhibition of P-450 activities, strongly indicated the occurrence of an enzyme in the liver microsomes that can transfer acetyl group from DAMC to the protein (P-450).

Although acetylation of P-450 apoprotein per se by DAMC was not demonstrated, the modification (by acetylation) of lysine of P-450 active center was thought plausible, similar to the mode of action of compounds such as chloramphenicol,[15] albeit the latter needed initial oxidative metabolism. Further studies on the mechanism of action of DAMC strengthened the role of microsomal TA in modification of other enzyme proteins resulting in the alteration of their catalytic activities. Accordingly, DAMC through the action of TA, was shown to irreversibly activate microsomal NADPH-cytochrome C reductase.[4] An elegant assay procedure was developed for the DAMC:protein transacetylase based on the irreversible inhibition of GST by DAMC.[11] In this communication, we have extended investigations on monoacetoxycoumarins with a view to examine the influence of the position of the acetoxy group on the benzenoid as well as the pyranone rings of the coumarin system governing the specificity to microsomal TA. For this purpose, coumarins **2**–**7** bearing one acetoxy group (Fig. 1), separately at positions C-7, C-6, C-5, C-4 and C-3 were synthesized and characterized as described under 'Materials and Methods' and their specificities to rat liver microsomal TA were examined. The results tabulated in Table 1 reveal that the preincubation of AC with liver microsomes and cytosol resulted in the inhibition of cytosolic GST. It is evident from the data that DAMC (1), because of possessing two acetoxy groups is a more effective substrate causing higher degree of GST inhibition compared to the monoacetoxycoumarins **2**–**7**. The specificity for TA action on various AC is in the order DAMC > 7-AMC > 6-AMC > 5-ADMC = 5-AMC = 4-AC > 3-AC (Table 1). To achieve a comparable level of GST inhibition, which is proportional to the enzymatic transfer of acetyl groups from AC to the protein (GST), the concentrations of DAMC, 7-AMC, 6-AMC, and 5-ADMC, 5-AMC, 4-AC and 3-AC required were in the order 1:2:4:8, respectively (Table 1). It is clear from the results that negligible TA activity was discernible when 3-AC was used as the substrate. This observation leads to the logical conclusion that the proximity of the acetoxy group on the benzenoid ring of the coumarin moiety to the oxygen heteroatom confers on the TA a high degree of acetyl group transfer capability. These results have amply clarified that acetoxy groups at C-7 and C-8 positions play a major role in the TA mediated transfer of acetyl groups to the protein(s). The acetoxy groups at other

Table 1. Rat liver microsomal acetoxycoumarin : protein transacetylase substrate specificity[a]

Time of preincubation	Transacetylase units[b]						
(min)	DAMC (25 μM)[c]	7-AMC (50 μM)	6-AMC (100 μM)	5-ADMC (200 μM)	5-AMC (200 μM)	4-AC (200 μM)	3-AC (200 μM)
10	8.2	7.7	6.4	5.2	6.0	5.5	2.0
20	15.9	15.0	11.5	9.6	11.9	9.9	4.2
30	24.4	21.1	23.9	16.7	17.3	14.9	5.7
40	30.2	26.4	29.9	21.6	22.4	17.0	7.7

[a]Acetoxycoumarins were preincubated with liver microsomes and liver cytosol, followed by the assay of GST as described under the 'Materials and Methods'. The unit of TA was expressed in terms of percent inhibition of GST under the conditions of the assay. The values are mean of three separate experiments with variation < 5%.
[b]Transacetylase unit is expressed in terms of percent inhibition of cytosolic GST under conditions of the assay.
[c]The numbers in the parentheses indicate the final concentration of AC in the assay system.

Table 2. Kinetic constants for acetoxycoumarin: protein transacetylase[a]

Substrate	K_m (μM)	ν_{max} (units)
DAMC	1000	364
7-AMC	1620	210
6-AMC	1902	145
5-ADMC	2385	92
5-AMC	2401	88
4-AC	2500	71
3-AC	2631	57

[a]The details are given under 'Materials and Methods'. The values are mean of three observations with variation < 5%.

carbon atoms on the benzenoid ring, as well as on the pyranone ring contribute very little to the catalytic action of TA. This conclusion is corroborated by the demonstration of higher specificity of coumarins to TA having acetoxy group on the benzenoid ring nearer to the oxygen heteroatom according to results tabulated in Table 2, which reveal K_m value of TA for DAMC < 7-AMC < 6-AMC < 5-ADMC = 5-AMC and ν_{max} values in the reverse order. We have further observed that in DAMC, the C-7 acetoxy group helps the C-8 acetoxy group to orient itself most favourably towards the oxygen heteroatom of the coumarin moiety as highlighted by the optimized structures (Fig. 2a,b). The relative orientation of acetoxy group towards the oxygen heteroatom is maximum in case of 7-AMC and the least in the case of 3-AC (Fig. 2b,c) and this phenomenon, presumably controls the TA mediated transfer of acetyl group to the protein(s). Transacetylase mediated irreversible activation of microsomal NADPH cytochrome C reductase by DAMC is depicted in Table 3, preincubation of microsomes in presence of DAMC, followed by the reductase assay revealed remarkable irreversible activation of the enzyme. The pattern of the reductase modulation by several monoacetoxycoumarins closely resembles that of transacetylase with 7-AMC effecting highest activation, the least being 3-AC. The efficacy of DAMC to stimulate the reductase is almost double than that of 7-AMC (Table 3). In an earlier report,[4] we had shown that DHMC, the deacetylated product of DAMC failed to elicit irreversible activation of reductase and also that the activation due to DAMC was completely abolished by *p*-hydroxymercuribenzoate. Based on these findings, the possible action of TA on DAMC in transferring the acetyl groups to the amino acid residues at the active center of the reductase (protein acetylation) resulting in the hyperbolic activation of the enzyme was postulated.[4] It is worth noting that low concentration of AC is sufficient to stimulate the reductase. Transacetylase-mediated inhibition of P-450 by DAMC as mentioned earlier was confirmed by the biological action of monoacetoxycoumarins. The results tabulated in Table 4 indicate the comparative abilities of several monoacetoxycoumarins to inhibit AFB_1 binding to calf thymus DNA in vitro in the order similar to their specificities to TA, i.e. 7-AMC > 6-AMC > 5-AMC. The inhibition of AFB_1-DNA binding in vitro by various AC (Table 4) is in tune with their specificities to TA, thus validating our conclusions on the irreversible inhibition of P-450 linked MFO

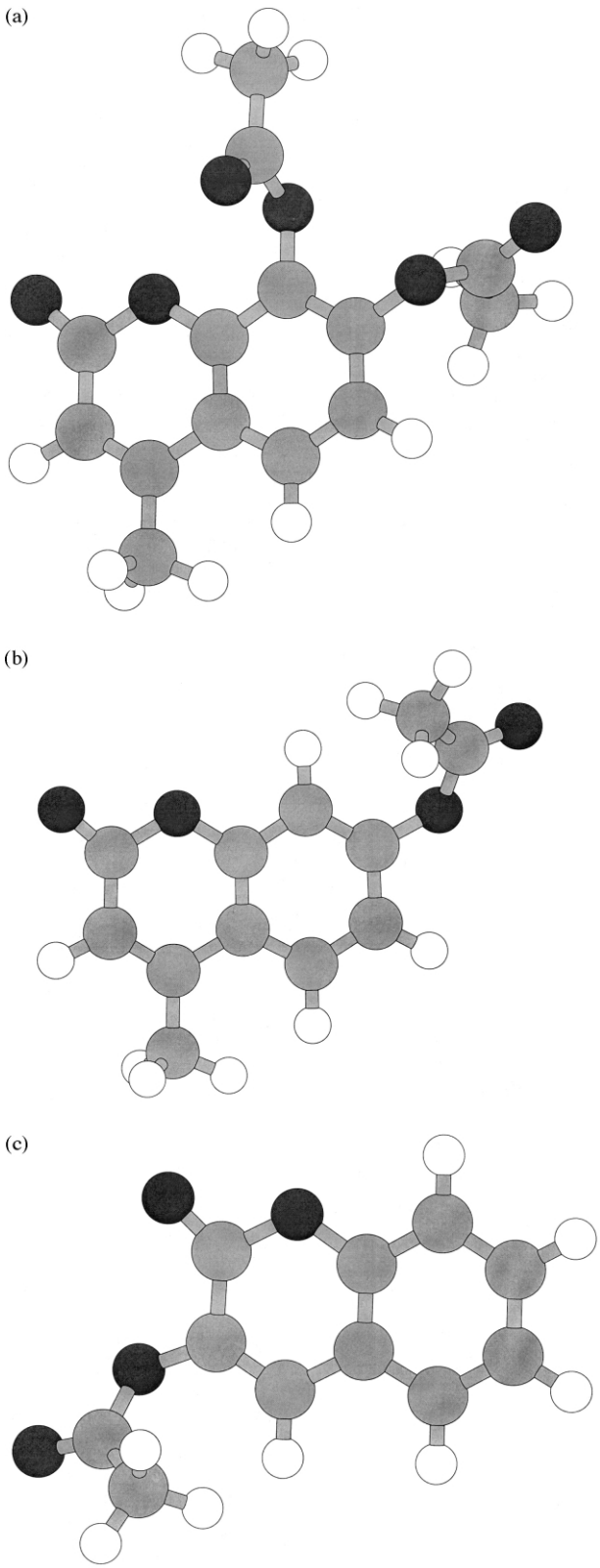

Figure 2. Optimized structures of acetoxycoumarins: (a) 7,8-Diacetoxy-4-methylcoumarin (DAMC); (b) 7-Acetoxy-4-methylcoumarin (7-AMC); (c) 3-Acetoxycoumarin (3-AC).

(Tables 1 and 2). This observation further substantiates our seminal finding that protein acetylation, in general by the acetylated drug can possibly be mediated through the action of TA. Transacetylase catalysed transfer of

Table 3. Irreversible activation of rat liver microsomal NADPH-cytochrome C reductase by acetoxycoumarins: structure–activity relation[a]

Time of preincubation (min)	Percent activation of NADPH cytochrome C reductase[b]						
	DAMC (2 μM)[c]	7-AMC (2 μM)	6-AMC (5 μM)	5-ADMC (5 μM)	5-AMC (5 μM)	4-AC (10 μM)	3-AC (10 μM)
5	10.2	5.8	7.15	4.50	5.1	7.6	5.1
10	18.9	11.9	14.90	9.25	10.5	14.9	9.5
20	38.9	24.4	25.40	16.80	17.8	28.6	21.0
30	57.6	35.2	42.0	25.95	27.2	37.7	26.3

[a]Acetoxycoumarins were preincubated with microsomes, followed by the addition of NADPH and cytochrome C for the assay of NADPH cytochrome C reductase. Percent activation of reductase was assessed compared to control as described under 'Materials and Methods'.
[b]The values represent average of three observations with error less than 2%.
[c]The numbers in the parentheses indicate final concentration of the acetoxycoumarin (AC) during their preincubation with liver microsomes.

Table 4. Modulation of aflatoxin-B_1 binding to rat liver DNA in vitro by acetoxycoumarins

Inhibitor	AFB_1-DNA binding *p*-moles AFB_1 bound/mg DNA/30 min[a]	% of inhibition
DAMC	85.1	58.2
7-AMC	128.7	36.7
6-AMC	145.6	28.4
5-ADMC	168.2	17.3
5-AMC	166.1	18.4
4-AC	184.6	9.3
3-AC	195.6	3.9
Control	203.5	—

[a]Values are average of three observations with variation $<5\%$. The details are given under 'Materials and Methods'.

acetyl group from one small molecule to another small molecule is known. The examples are the enzymatic transfer of (a) acetyl group from platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, alkylacetyl-GPC) to lysoplasmalogen,[16,17] and (b) transacetylase catalysing acetyl CoA independent acetylation of aryl amines.[18,19] The existence of a membrane bound TA catalyzing the transfer of acetyl group from a foreign compound (small molecule) to proteins resulting in the modulation of their (protein) functions, as exemplified and demonstrated by the action of AC in this paper and in our previous reports[1–4,11] presents a unique example of protein acetylation hitherto not reported. Further experiments involving isotopically labelled substrates and analysis of the postulated acylated GST formed in these studies by MALDI/LCMS techniques to confirm these findings are in progress and the results shall be presented in a future communication.

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