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Synthesis of Novel 4-Methylcoumarins and Comparative Specificities of Substituted Derivatives for Acetoxy Drug: Protein Transacetylase

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Abstract

Our laboratory has been credited for the discovery of a unique membrane bound enzyme termed Acetoxy Drug: Protein Transacetylase (TAase) catalyzing the transfer of acetyl group from polyphenolic peracetates (PA) to certain functional proteins resulting in the modulation of their catalytic activity. In this report, we have synthesized eight novel 4-methylcoumarins and demonstrated the comparisons of acetoxy derivatives of 4-methylcoumarin with their propoxy and butoxy derivatives for the modulation of some receptor proteins such as cytochrome P-450 (Cyt.P-450), NADPH cytochrome c reductase and cytosolic glutathione S-transferase (GST). The results clearly indicated that acetoxy derivatives have very high efficacy for the modulation of above mentioned functional proteins as compared to their other derivatives. We have also compared the acetoxy derivatives of 4-methylcoumarin with their acid substituted acetoxy derivatives and found that inclusion of carboxylic acid groups on the benzenoid rings of the coumarins system hardly affected TAase mediated catalytic activity.

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Keywords

Coumarins • Acetoxy drug: protein transacetylase (TAase) • DAMC • Structure Activity Relationship (SAR) • Acetyl group.

Introduction

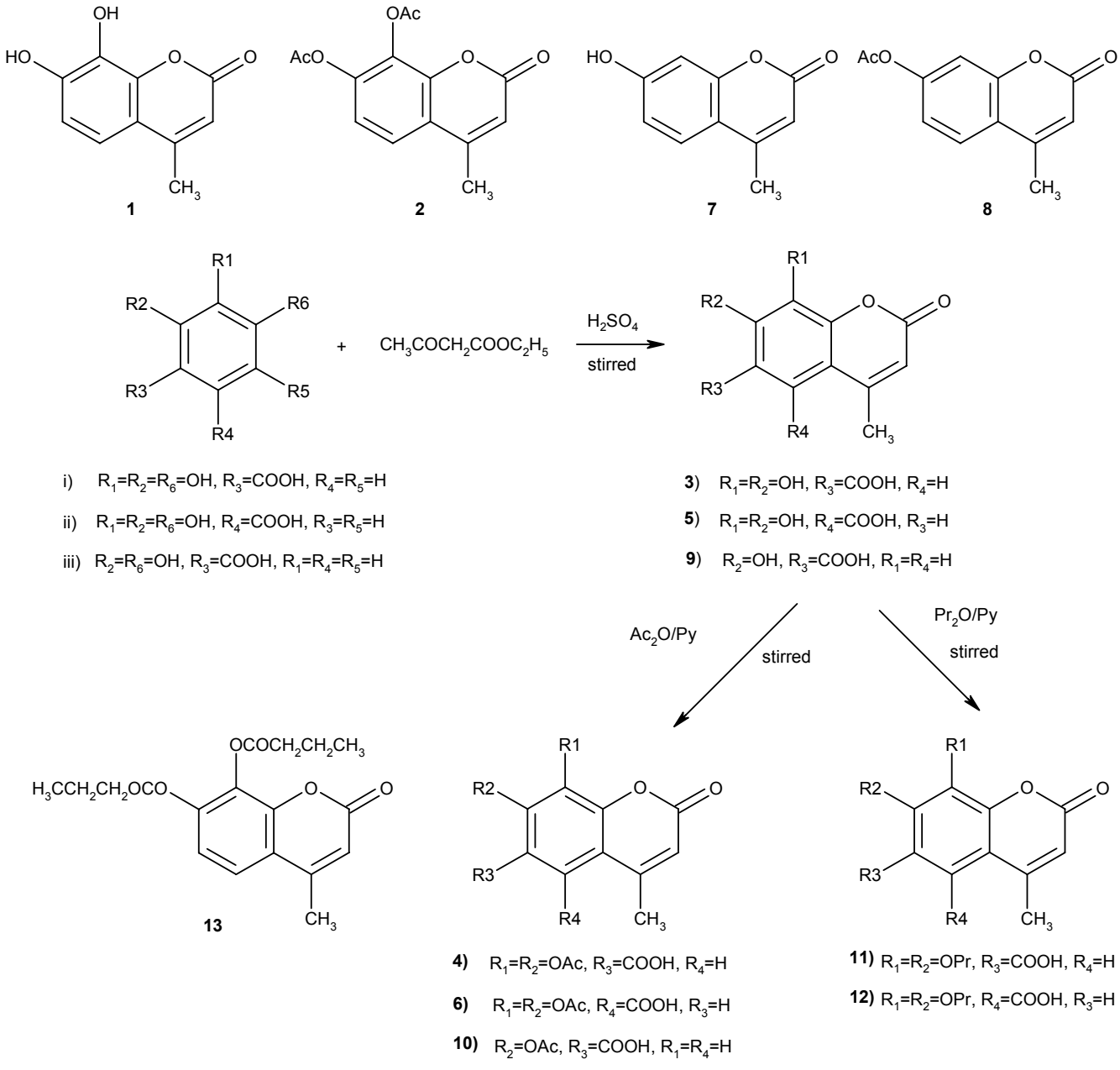
In our earlier publications [1, 2] evidences were given for the existence of a novel enzyme acetoxy drug: protein transacetylase (TAase) present in rat liver microsomes. The enzyme, transacetylse catalyzed the transfer of acetyl group from the various acetoxy drugs to specific enzyme proteins such as cytochrome P-450 (Cyt. P-450) [3], NADPH cytochrome c-reductase [4] and glutathione S-transferase [1, 2, 5]. The simple assay procedures were developed by modifications in the literature procedures for the liver microsomal transacetylase assays [1]. The TAase assay procedure was based on the irreversible inhibition of GST (protein substrate) with the use of various acetoxy derivatives of heterocyclic molecules (second substrates) [1, 2]. One of the products of the transacetylase reaction was identified as the hydroxyl derivatives of the respective acetoxy derivatives [1]. The functional protein, NADPH cytochrome c-reductase assay was based on the activation of an enzyme participating in hepatic microsomal electron transport and Cyt. P-450 assay was based on the inhibition of liver microsomal Cyt. P-450, linked mixed function oxidases (MFO) by acetoxy derivatives of heterocyclic molecules catalyzing the epoxidation of aflatoxin B_1 (AFB_1) [3]. Apart from these activities we have also used the polyphenolic peracetates (PA) to modify another protein called Nitric Oxide Synthase (NOS). As enhanced intracellular levels of Nitric Oxide (NO) helps vasorelaxation in asthmatic patients. The intracellular level of NO alteration is controlled by the mechanism involved in the activation of NOS. Our studies, for the first time highlighted the role of PA as the novel potent agent for enhancing the intracellular levels of NO [6]. We had also demonstrated TAase catalyzed modification of Protein Kinase C (PKC) activity in lymphocytes of asthmatic patients by PA. The PKC activity of lymphocytes of asthmatic patients was noticed to

proportionally increase with severity of the disease [7]. Acetoxy derivatives were found to exert inhibitory action on PKC while hydroxyl derivatives of the respective compounds did not show any activity. We had also highlighted the activation of human platelets NOS by PA catalyzed by Transacetylase (TAase) [6]. Purified human placental TAase mediated acetylation of nNOS by DAMC was demonstrated [8]. It was thought interesting to examine more and more acetoxy drugs to find out a better substrate for the transacetylase mediated biological actions. In this connection, eight novel 4-methylcoumarins were synthesized and characterized by spectral data (UV, IR, ^{1}H NMR and ^{13}C NMR) to examine structure activity relationship (SAR) of acetoxy, propoxy and butoxy derivatives of 4-methylcoumarin on liver microsomal Cyt. P-450 linked MFO, NADPH cytochrome c-reductase and cytosolic glutathione S-transferase (GST).

Results and Discussion

The 4-methylcoumarins (**3–6** & **9–12**) were synthesized by the well known Pechmann condensation [9] in quantitative yield. The compound **3–6** & **9–12** are outlined in scheme 1.

Structure of the compounds was confirmed by the ^{1}H NMR, ^{13}C NMR, UV, IR and data for these compounds are given in the chemistry section. The IR spectrum of compound **3** showed two characteristic absorptions at 3751 and 3304 cm^{-1} for hydroxyl groups and two other absorptions appeared at 1769 and 1699 cm^{-1} for carbonyl groups. The ^{1}H NMR spectrum showed characteristic peak of 4-methylcoumarin at δ 6.91 for C-3 proton. Finally the structure was supported by its ^{13}C NMR spectrum, C-2 appeared at δ 158.28 and other carbonyl carbon appeared at δ 167.80 for COOH. Compound **4** had two characteristic absorptions at 3368 and 1701 cm^{-1} for hydroxyl and carbonyl groups respectively in its IR spectrum. The ^{1}H NMR spectrum showed characteristic singlet of 4-methylcoumarin at δ 6.21 for C-3 proton. Finally the structure was confirmed by its ^{13}C NMR spectrum, C-2 appeared at δ 158.12 and three other carbonyls were



Sch. 1. Synthetic route for the synthesis of 4-methylcoumarin derivatives. All the compounds were characterized by spectral data (UV, IR, ^{1}H NMR, ^{13}C NMR)

appeared at δ 166.75, 167.11 and 169.95. Compound **5** showed two characteristic absorptions at 3394 and 1699 cm^{-1} for hydroxyl and carbonyl group respectively in IR spectrum. A characteristic peak of 4-methylcoumarin appeared at δ 6.14 for C-3 proton in ^{1}H NMR spectrum. Finally the structure was supported by ^{13}C NMR spectrum, two carbonyl peaks appeared at δ 160.56 and 172.0 for C-2 and COOH

carbons respectively. The IR spectrum of compound **6** showed absorption at 3619 cm^{-1} for COOH group and two other absorptions appeared at 1780 and 1699 cm^{-1} for carbonyl carbons. In its ^{1}H NMR spectrum, a characteristic peak of 4-methylcoumarin appeared at δ 6.32 for C-3 proton. Finally the structure was confirmed by its ^{13}C NMR spectrum, C-2 appeared at δ 158 and carbonyl for COOH appeared at δ 200.26. The IR spectrum of compound **9** showed three characteristic absorptions at 3675, 3447 and 1702 cm^{-1}, two for hydroxyl group and one for carbonyl group respectively. In its ^{1}H NMR spectrum, a characteristic peak of 4-methylcoumarin was appeared at δ 6.20 for C-3 proton. Finally the structure was confirmed by its ^{13}C NMR spectrum, C-2 appeared at δ 157.99 and other carbonyl carbons appeared at δ 164.68. Compound **10** had two characteristic absorption at 3476 and 1699 cm^{-1} for hydroxyl and carbonyl groups respectively in its IR spectrum. The ^{1}H NMR spectrum showed characteristic peak of 4-methylcoumarin at δ 6.20 for C-3 proton. Finally the structure was confirmed by its ^{13}C NMR spectrum, C-2 appeared at δ 158.86 and two other carbonyl carbons appeared at δ 162.59 and 166.64. Compound **12** had three characteristic absorptions at 1792, 1761, 1699 cm^{-1} for acetoxy, carboxylic and C-2 carbonyl groups respectively in its IR spectrum. The ^{1}H NMR spectrum showed characteristic singlet of 4-methylcoumarin, appeared at δ 6.31 for C-3 proton. Finally the structure was confirmed by its ^{13}C NMR spectrum, C-2 appeared at δ 158.45 and other carbonyls appeared at δ 161.16 and 166.16. The IR spectrum of compound **11** showed two characteristic absorptions which appeared at 3394 and 1700 cm^{-1} for hydroxyl and carbonyl groups respectively. The ^{1}H NMR spectrum showed characteristic singlet of 4-methylcoumarin at δ 6.31 for C-3 proton. Finally the structure was supported by its ^{13}C NMR spectrum, CH_2 carbon appeared at δ 62.24 and C-2 appeared at δ 158.80. The other carbonyl groups appeared at δ 163.68 and 172.21.

For the structure activity relationship, number of acetoxy, propoxy and butoxy derivatives of 4-methylcoumarin were synthesized and examined with the liver microsomal Transacetylase (TAase) for comparative specificities of acyl groups of coumarin derivatives. The catalytic activity of TAase was governed by carbonyl

group in pyran ring of coumarin nucleus [2]. For the TAase catalytic activity, the inhibition of GST under the conditions of assay was considered to be proportional to the TAase activity as demonstrated in our earlier publications [1, 2, 5]. The results in table 1 show the TAase catalyzed inhibition of cytosolic GST by polyphenolic acetates, 7,8-diacetoxy-4-methylcoumarin-5-carboxylic acid (**4**) and 7,8-diacetoxy-4-methyl-coumarin-6-carboxylic acid (**6**) causes significant inhibition of TAase which is quite identical to that of the model acetoxy drug **2**. The extent of inhibition of GST by 7-acetoxy-4-methylcoumarin-6-carboxylic acid (**10**) is similar to that of the 7-acetoxy-4-methylcoumarin (**8**). These results clearly indicate that the addition of carboxylic group on the benzenoid ring hardly affect on TAase mediated catalytic activity. The transfer of acetyl group from polyphenolic acetates to receptor proteins catalyzed by the novel enzyme acetoxy drug: protein TAase has also been established by our earlier studies [10, 11]. DAMC, **2** a model acetoxy drug 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 [3, 4]. Our previous studies have highlighted the structural features of poly-phenolic acetates determining the specificity for liver microsomal TAase [12, 13].

The acetoxy coumarins were also examined for the ability to irreversibly activate liver microsomal NADPH cytochrome c reductase, which is taken as another yardstick to assess the activity and specificity of TAase (Table 2). It is interesting to note that various concentrations of acetoxy coumarins cause the activation of the reductase (catalyzed by TAase) in tune with their specificities of TAase measured in terms of inhibition of cytosolic GST.

These coumarin derivatives were also used as substrates for the inhibition of liver microsomal catalyzed AFB_1 binding to DNA in vitro. The pattern of inhibition of AFB_1-DNA binding by acetoxy derivatives of 4-methylcoumarin and their acid containing acetoxy derivative followed similar trend as observed earlier. Accordingly, compound **2**, **4** and **6** inhibited liver microsomal cytochrome P-450 catalyzed AFB_1 epoxidation (measured as binding to DNA) to a similar extent. Similar trend also persisted for compounds **8** and **10** (Table 3).

Tab. 1. Relative specificities of various derivatives of 4-methylcoumarin to liver microsomal acetoxy drug: protein transacetylase

Test compounds	Concentration (μM)	TAase catalytic activity	Standard deviation
2	25	8.08	0.030
4	25	7.69	0.200
6	25	8.11	0.100
8	50	7.65	0.311
10	50	7.80	0.092
11	50	6.81	0.034
12	50	6.66	0.140
13	100	5.88	0.051

Comparison of relative specificity to acetoxy/propoxy/butoxy derivatives of 4-methylcoumarins to liver microsomes to acetoxy drug: Protein transacetylase.
Units of TAase activity expressed in terms of % inhibition of GST under condition of the assay.
Values are mean of three observation with variation <5%
Number in bold letters denotes the test compound and preincubated for 10 min.

Tab. 2. Activation of liver microsomal NADPH cytochrome c reductase by various derivatives of 4-methylcoumarin catalyzed by microsomal acetoxy drug: protein transacetylase

Test compound	Concentration (μM)	Activation of NADPH cytochrome C-reductase	Standard deviation
2	2	18.10	0.150
4	2	17.50	0.150
6	2	17.75	0.030
8	2	10.99	0.185
10	2	10.21	0.026
11	5	13.52	0.020
12	5	13.65	0.685
13	5	6.21	0.260

Activation of liver microsomal NADPH cytochrome c reductase by polyphenolic acetates catalyzed by microsomal acetoxy drug: Protein transacetylase.
Values are mean of three observations with variation less than <5%.
Number in bold letters denotes the test compound and preincubated for 10 min.

Tab. 3. Inhibition of liver microsomes catalyzed AFB_1-DNA binding in vitro by substituted 4-methylcoumarins

Test Compounds	AFB_1-DNA binding p-moles AFB_1 bound/mg DNA/30 mins	% Inhibition	Standard deviation
2	80.1	58.5	0.520
4	85.1	58.2	0.304
6	89.2	59.5	1.450
8	128.7	33.0	0.577
10	132.2	30.4	0.945
11	170.2	10.4	0.450
12	168.2	11.5	0.604
13	352.2	3.2	0.152

Comparison of TAase catalyzed modulation of rat liver microsomes mediated aflatoxin B_1 binding to DNA in vitro by acetoxy/propoxy derivatives of 4- methylcoumarin. Inhibition of AF B_1 binding to DNA (Pmoles/mg/30 min). Concentration of test compound was 100 µM.

Values are mean of three observations with variation <5%.

Number in bold letters denotes the test compound number.

The TAase-mediated inhibition of liver microsomal cytochrome P-450 linked mixed function oxidase (MFO) by acetoxy derivative of 4-methylcoumarin is shown in Table 3, 4 and 5. For this purpose, test compounds were preincubated with liver microsomes, followed by the addition of ethyoxyresorufin/pentoxyresorufin and NADPH in order to assay the activity of ethoxyresorufin deethylase (EROD) and pentoxyresorufin (PROD). The results are shown in Table 4 and 5. These results conclusively ratify the inferences drawn earlier on the specificity of these compounds to TAase.

Tab. 4. Effect of substituted 4-methylcoumarins on the liver microsome catalyzed dealkylation of alkylated resorufin (Ethoxyresorufin)

Test Compounds	% Inhibition (unit/min.)	Standard deviation
2	45.22	0.087
4	40.11	1.050
6	41.25	0.866
8	25.27	0.214
10	27.15	1.003
11	26.15	0.150
12	26.52	0.551
13	15.12	0.866

Tab. 5. Effect of substituted 4-methyl coumarins on the liver microsomes catalyzed dealkylation of alkylated resorufin (Pentoxyresorufin)

Test Compounds	% Inhibition (unit/min.)	Standard deviation
2	36.05	0.264
4	34.2	0.779
6	35.0	1.005
8	26.2	0.303
10	27.1	0.200
11	32.2	1.113
12	28.0	0.813
13	12.96	0.515

Inhibition of liver microsomal cytochrome P-450 linked EROD/PROD by acetoxy/propoxy derivatives of 4-methhylcoumarin catalyzed by acetoxy drug: Protein transacetylase. Concentration of test compound dissolved in DMSO was 25 μM.
Values are mean of three observations with variation <5%.
Number in bold letters denotes the test compound number and preincubated for 10 min.

We have also examined propoxy and butoxy derivative of 4-methylcoumarin for the first time for the modulation of above described functional proteins. The results presented in Table 1-5 (compounds 7,8-dipropoxy-4-methylcoumarin-5-carboxylic acid, **11** 7,8-dipropoxy-4-methylcoumarin-6-carboxylic acid, **12** and 7,8-dibutoxy-4-methylcoumarin, **13**) clearly indicate that propoxy compounds showed less degree of modulation and butoxy derivative exhibited much lesser degree of modulation as compared to acetoxy derivative in the modulation of aforesaid functional proteins. These observations clearly indicated that the compound containing acetoxy groups could actively facilitate the TAase catalyzed transfer of acetyl groups to the proteins while the propoxy and butoxy groups at the same positions (C-7 and C-8) on the benzenoid ring contribute less to the TAase catalytic activity because of transfer of propoxy and butoxy group from propoxy and butoxy derivatives to proteins with slower rate as compared to acetoxy derivatives which in turn showed less activity. Here it can be explained that as the numbers of carbon atoms in the acyl group at 7^{th} and 8^{th} position of benzenoid ring increases, the steric hindrance at carbonyl group of acyl group increases. As a result the tendency of attacking the carbonyl group by nucleophilic amino group of lysine residues of our enzyme decreases. Thus the acetoxy derivatives are better substrates for TAase than propoxy and butoxy derivatives.

Further, the obtained results supported by PM3 optimized structure, suggested that the electron charge distribution and bond order about the active O-Ac bond nearly remain same in all the systems considered here. The longest axis measurement (6.86 cm) for the PM3 optimized geometry of DAMC (2) is almost equal to 7,8-diacetoxy coumarin-6-carboxylic acid (6.952 cm) and 7,8-diacetoxy coumarin-5-carboxylic acid (6.950 cm) is found to be much smaller than that of 7,8-dipropoxy 4-methylcoumarin (9.4 cm) and 7,8-dibutoxy-4-methylcoumarin (10.966 cm). This is in agreement with an observed result that compounds **2**, **4** and **6** shows higher TAase catalytic activity as compared to compounds **11**, **12** and **13**. This is because of the possible hindrance to the propoxy and butoxy groups from the accessing the active site of TAase resulting in the decreased rate of transfer of the

propyl and butyl groups to the enzyme. Further the PM3 optimized geometries have revealed that the acetoxy groups in compound **2**, **4** and **6** are bent towards the oxygen heteroatom of the pyran ring. We have further observed that in compound **2**, **4** and **6**, the C-7 acetoxy group helps the C-8 acetoxy group to orient itself most favorably towards the oxygen heteroatom of the coumarins moiety and this phenomenon, presumably controls the TAase mediated transfer of acetyl group to the functional proteins.

Experimental

Chemicals

Reduced glutathione (GSH), NADPH, cytochrome c, aflatoxin B_1 (AFB_1), 1-chloro-2,4-dinitrobenzene (CDNB) and calf thymus DNA were purchased from Sigma Company, ST Louis MO (USA). [^{3}H] AFB_1-(G) was obtained from Moravek Biochemicals, Brea, CA (USA). Ethoxyresorufin and pentoxyresorufin were purchased from Fluka Chemicals. All other chemicals were of high grade and were procured from local suppliers.

General

Melting points were determined in sulphuric acid bath and were uncorrected. The UV and IR Spectra were recorded on Beckman DU-64 Spectrophotometer and Perkin-Elmer model 1720 FTIR spectrophotometer respectively. The ^{1}H NMR spectra were recorded either at Shimartzu or Bruker AC-250 instrument at 60 and 300 MHz respectively. The ^{13}C NMR spectra were recorded on same instrument as ^{1}H NMR spectral recordings at 62.8 and 75 MHz respectively. TMS has been used as an internal standard for both ^{1}H and ^{13}C NMR spectral recordings. The chemical shift values are on δ scale. The mass spectra were recorded on a Varian Mat 311, a mass spectrometer at 70 eV. The physical and spectral data of DHMC and DAMC have been published previously [14].

Chemistry

Known 4-methylcoumarin derivatives

The 7,8-dihydroxy-4-methylcoumarin (**1**) and 7-hydroxy-4-methylcoumarin (**7**) were prepared by well known Pechmann condensation of pyragallol and resorcinol with ethylacetoacetate [9], the acetoxy derivatives 7,8-diacetoxy-4-methylcoumarin (**2**), 7-acetoxy-4-methylcoumarin (**8**) and butoxy derivatives 7,8-dibutoxy-4-methylcoumarin (**13**), were synthesized by the acetylation and butylation of their hydroxyl derivatives with acetic anhydride and butyric anhydride with pyridine and characterized according to the data reported in literature [14].

*7,8-Dihydroxy-4-methyl-2-oxo-2***H***-chromene-5-carboxylic acid (3)**

Prepared as white colored powder from 3,4,5-trihydroxy benzoic acid (5.8 mM) by procedure described in compound **5**. Crystallized from ethanol (50% yield).; MP: >260 ^{0}C; UV (MeOH): λmax 320, 245 nm.; IR (Nujol): Vmax 3751, 3304, 2956, 2724, 2365, 1790, 1769, 1699, 1616, 1593, 1460, 1376, 1204, 1165, 1050, 964, 893,851 cm^{-1}.; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.47 (3H, s, CH_3), 6.91 (1H, s, C-3H) and 9.11 (1H, s, C-6H).; ^{13}C NMR (75 MHz, $CDCl_3$): δ 20.12 (C-4CH_3), 109.07 and 111.21 (C-3, C-6), 119.20 and 120.79 (C-9, C-10), 138.32 (C-4), 145.71, 146.23 and 148.45 (C-5, C-7, C-8), 158.28 (C-2, C=O), 167.80 (*C*OOH).

*7,8-Bis(acetyloxy)-4-methyl-2-oxo-2***H***-chromene-5-carboxylic acid (4)**

White colored powder from compound **3** (0.04 mM), prepared as described in the preparation of compound **6**. Crystallized from chloroform (80% yield).; MP: 230 ^{0}C; UV (MeOH): λmax 267 nm.; IR (Nujol): Vmax 3368, 2924, 2724, 1701, 1618, 1541, 1460, 1377, 1266, 1169, 1028, 867 cm $^{-1}$.; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.30 (9H, s, CH_3, 2xOCO*C*H_3), 6.21 (1H, s, C-3H) and 7.56 (1H, s, C-6H).; ^{13}C NMR (75 MHz, $CDCl_3$): δ 20.53 and 20.93 (C-4CH_3 and 2xOCOC*H*$_3$), 115.12 and 116.17 (C-3, C-5), 123.21 and 127.78 (C-9, C-10), 139.76 (C-4), 143.93, 145.16 and 148.67 (C-6, C-7, C-8), 158.12 (C-2, C=O), 166.75 and 167.11 (2x*O*COCH$_3$), 169.95 (*C*OOH).

7,8-Dihydroxy-4-methyl-2-oxo-2*H*-chromene-6-carboxylic acid (5)

7,8-dihydroxy-4-methylcoumarin-6-carboxylic acid (**5**) was prepared by the Pechmann condensation of 2,3,4-trihydroxy benzoic acid with ethyl acetoacetate. To an ice cold H_2SO_4 (5.0 ml), solution of 2,3,4-trihydroxy benzoic acid (1.0 gm., 0.0058 mM) in ethyl acetoacetate (2.8 ml, 0.0174) was added slowly with constant stirring. After completion of the addition, reaction mixture was stirred for 6 hrs at room temperature and the progress of the reaction was monitored on TLC. Aqueous work up of the reaction mixture yielded white colored product of m.p. 253 ^{0}C (yield 40%) which was crystallized by ethanol to obtain pure compound. Crystallized from ethanol (40% yield).; MP: 253 ^{0}C; UV (MeOH): λmax 320, 249 nm.; IR (Nujol): Vmax 3619, 3394, 2724, 2364, 1868, 1772, 1699, 1653, 1559, 1459, 1376, 1205, 1167, 1052, 973, 854 cm$^-$.; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.41 (3H, s, CH_3), 6.14 (1H, s, C-3H) and 7.68 (1H, s, C-5H).; ^{13}C NMR (75 MHz, $CDCl_3$): δ 19.16(C-4CH_3), 110.45 and 112.76 (C-3, C-5), 113.26 and 117.49 (C-9, C-10), 133.61 (C-4), 146.54 and 153.68, 153.77 (C-6, C-7, C-8), 160.56 (C-2, C=O), 172.02 (COOH).

7,8-Bis(acetyloxy)-4-methyl-2-oxo-2*H*-chromene-6-carboxylic acid (6)

The acetylated compound 7,8-diacetoxy-6-carboxylic-4-methylcoumarin (**6**) was prepared by the acetylation of compound **5** (0.0 4mM) by stirring it at room temperature for 5 hrs with acetic anhydride (0.08 mM) and pyridine (0.06 mM) and the progress of the reaction was monitored on TLC using ethyl acetate: petrol (25:75). After completion of the reaction, reaction mixture was poured over crushed ice. The solid that precipitated out was filtered, dried over P_2O_5 in a vacuum desiccator and the crude product was purified by crystallization and afforded the compound 6 in crystallized form. Crystallized from chloroform (80% yield).; MP: 212 ^{0}C; UV (MeOH): λmax 217, 240 nm.; IR (Nujol): Vmax 3619, 2921, 2724, 2365, 1780, 1699, 1627, 1539, 1567, 1458, 1376, 1241, 1170, 1081, 917, 817 cm^{-1}.; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.35, 237 and 2.41 (9H, 3s, CH_3, 2xOCOCH_3), 6.32 (1H, s, C-3H) and 7.50 (1H, s, C-5H).; ^{13}C NMR (75 MHz, $CDCl_3$): δ 18.98, 20.43 and

20.95 (C-*4*CH_3 and 2xOCO*C*H_3), 109.10 and 11.62 (C-3, C-5), 113.38 and 11.40 (C-9, C-10), 133.10 (C-4), 122.46, 125.53 and 126.29 (C-6, C-7, C-8), 158.00 (C-2, C=O), 167.11 (2x*O*COCH_3), 200.26 (*C*OOH).

*7-Hydroxy-4-methyl-2-oxo-2*H*-chromene-6-carboxylic acid (9)*

Prepared as white colored powder from 2,4-dihydroxy benzoic acid (0.04 mM), by procedure described in the preparation of compound **5**. Crystallized from ethanol (60% yield).; MP : 217 ^{0}C; UV (MeOH): λmax 324, 320 nm.; IR (Nujol): Vmax 3447, 3675, 2925, 2724, 2365, 1868, 1829, 1702, 1103, 1684, 1616, 1540, 1507, 1458, 1376, 1250, 1165, 935, 860 cm^{-1}.; ^{1}H NMR (300 MHz DMSO-d_6): δ 2.41 (3H, s, CH_3), 6.20 (1H, s, C-3H) and 6.83 (1H, s, C-5H), 8.10 (1H, s, C-8H).; ^{13}C NMR (75 MHz, $CDCl_3$): δ 18.32 (C-4CH_3), 104.04, 112.11 and 112.84 (C-3, C-5, C-8), 123.23 and 128.56 (C-9, C-10), 134.56 (C-4), 148.56 and 153.54 (C-6 C-7), 157.99 (C-2, C=O), 164.68 (COOH).

*7-(Acetyloxy)-4-methyl-2-oxo-2*H*-chromene-6-carboxylic acid (10)*

White colored powder from compound **11** (0.08 mM), prepared as described in the preparation of compound **6**. Crystallized from chloroform (30 % yield).; MP: 202 ^{0}C; UV (MeOH): λmax 277.; IR (Nujol) : Vmax 3476, 2924, 2723, 2364, 1772, 1734, 1699, 1653, 1576, 1507, 1458, 1376, 1224, 1165, 1060, 935, 884 cm^{-1}.; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.41 (3H, s, CH_3), 6.20 (1H, s, C-3H) and 6.83 (1H, s, C-5H), 8.10 (1H, s, C-8H).; ^{13}C NMR (75 MHz, $CDCl_3$): δ 18.86 and 21.14 (C-4CH_3 and OCO*C*H_3), 104.54, 110.07 and112.57 (C-3, C-5, C-8), 119.57 and 122.58 (C-9, C-10), 134.54 (C-4), 146.23 and 152.86 (C-6, C-7), 158.86 (C-2, C=O), 162.59 (O*C*OCH_3), 166.64 (COOH).

*4-Methyl-2-oxo-7,8-dipropoxy-2*H*-chromene-5-carboxylic acid (11)*

Prepared as white colored powder from compound **3** (0.08 mM), by procedure described in the preparation of compound **6**. Crystallized from chloroform (55 % yield).; MP: >215 ^{0}C; UV (MeOH): λmax 277, 249 nm.; IR (Nujol): Vmax 3394, 2932, 2724, 1752, 1733, 1700, 1684, 1569, 1459, 1303, 1158, 975, 854, 722 cm^{-1}.;

^{1}H NMR (300 MHz, $CDCl_3$): δ 2.48, 2.63 (9H, 2s, CH_3, 2xOCOCH$_2$C*H$_3$*), 4.33 (4H, q, 2xOCOC*H$_2$*CH$_3$), 6.31 (1H, s, C-3H) and 8.17 (1H, s, C-6H).; ^{13}C NMR (75 MHz, $CDCl_3$): δ 19.13, 27.49 27.79 (C-4CH_3 and 2xOCO CH_2CH_3), 62.24 (2xOCO*C*H$_2$CH$_3$), 115.83 and 118.62 (C-3, C-6), 120.84 and 125.14 (C-9, C-10), 134.34 (C-4), 143.93 , 146.56 and 149.05 (C-5, C-7, C-8), 158.80 (C-2, C=O), 163.68 (2xO*C*OCH$_2$CH$_3$), 172.21 (COOH).

*4-Methyl-2-oxo-7,8-dipropoxy-2*H*-chromene-6-carboxylic acid (12)*

The propoxy derivative of compound **5** (0.08 mM) was synthesized by the propylation with propionic anhydride (0.16 mM) and pyridine (0.12 mM) and the white colored product was obtained according to the procedure given in the preparation of compound **6**. Crystallized from chloroform (60 % yield).; MP: >260 °C; UV (MeOH): λmax 320, 249 nm; IR (Nujol): Vmax 3394, 2928, 2724, 1792, 1761, 1699, 1617, 1559, 1459, 1303, 1154, 1080, 973, 848, 722 cm^{-1}.; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.41 (9H, s, CH_3, 2xOCOCH$_2$C*H$_3$*), 4.35 (4H, q, 2xOCOC*H$_2$*CH$_3$), 6.31 (1H, s, C-3H) and 7.25 (1H, s, C-5H).; ^{13}C NMR (75 MHz, $CDCl_3$): δ 21.49, 29.85 and 30.15 (C-4CH_3 and 2xOCOCH$_2$*C*H$_3$), 64.45 and 75.13 (2xOCO*C*H$_2$CH$_3$), 118.28 and 120.98 (C-3, C-5), 123.20 and 127.50 (C-9, C-10), 133.14 (C-4), 145.57 and 151.78 and 154.55 (C-6, C-7, C-8), 158.45 *(*C**-**2, C=O), 161.16 (2xO*C*OCH$_3$), 166.16 (COOH).

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-mercaptoehtanol, pH adjusted to 7.0. The homogenate was centrifuged at 10,000g for 30 min. and the obtained supernatant was spun at 100,000 g for 1 h in the Beckman ultracentrifuge Model L-7. The cytosolic fraction was set-aside at -20 °C.

The microsomal pellet was resuspended in 0.25 M sucrose. Protein contents of microsomes and cytosol were estimated by the method of Lowry et al. [15].

Assay of acetoxy drug: protein transacetylase

The principle governing the TA assay and the detailed assay procedure were given in our earlier publication [1]. The assay was carried out using acetoxy drug 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), Acetoxy drug dissolved in DMSO (100 µM), liver cytosol (12.5 µg protein) and water to make up a total volume of 0.8 mL. The contents of the tubes (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 [1]. The units of TA present were expressed in terms of percent inhibition of GST under the conditions of the assay.

Assay for AFB_1 binding to DNA

Liver microsomes catalyzed AFB_1 binding to DNA *in vitro* and inhibition by acetoxy drugs was carried out as described in our earlier publications [3].

Assay for ethoxyresorufin O-dealkylase (EROD) and pentoxyresorufin O-dealkylase (PROD)

The assay of EROD/PROD was performed by the method of Lubet et al. [16].

Modulation of NADPH cytochrome C-reductase

The method consisted of preincubation of acetoxy drug 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 acetoxy drug dissolved in DMSO (5 µM), 0.005 M phosphate buffer (pH 7.7) and water to make 0.5 mL volume. The contents, scaled up as per the

requirement, were preincubated at 37 °C in a shaker 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 1mM 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, acetoxy drugs were replaced by DMSO. The increment in reductase activity due to acetoxy drug over the control was expressed as percent activation.

Statistical analysis

The final values are the mean of three observations and standard deviation was calculated with the help of following website:

www.easycalculation.com/statistics/standard-deviation

Optimization of structures of 7,8-diacetoxy-4-methylcoumarin (2), 7,8-diacetoxy-4-methylcoumarin-5-carboxylicacid (4), 7,8-diacetoxy-4-methylcoumarin-6-carboxylic acid (6), 7-acetoxy-4-methylcoumarin (8) 7-acetoxy-4-methylcoumarin-6- carboxylic acid (10)

A semi empirical procedure was used to study the enzyme mediated transacetylation reactions of various acetoxycoumarins. The molecular spatial structure and bond length analysis of these systems was theoretically estimated using HyperChem Release 5.1 Pro Quantum Chemistry Package [17]. The preliminary PM3 optimized structures were subjected to a systematic conformational search and the lowest energy conformation obtained was then fully optimized without geometric constraints using RHF/PM3 semi empirical methods [18].

Conclusion

Eight novel 4-methylcoumarins were synthesized and their structures were confirmed by the spectral data. The results presented in this communication revealed that the *ortho* diacetoxy system is better than those of *ortho* dipropoxy and

dibutoxy system with respect to catalytic activity of glutathione S-transferase, NADPH cytochrome c-reductase, AFB_1–DNA binding and cytochrome P-450 linked MFO. Introduction of carboxylic group at any position in the benzenoid ring of the coumarin moiety is ineffective in the modulation of aforesaid functional proteins.

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