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Calreticulin transacetylase: A novel enzyme-mediated protein acetylation by acetoxy derivatives of 3-alkyl-4-methylcoumarins

Sarah Jalal [a], Karam Chand [a], Abha Kathuria [a], Prabhjot Singh [b], Nivedita Priya [b], Bhavna Gupta [c], Hanumantharao G. Raj [b], Sunil K. Sharma [a,*]

[a] Bioorganic Laboratory, Department of Chemistry, University of Delhi, Delhi 110 007, India
[b] Department of Biochemistry, VP Chest Institute, University of Delhi, Delhi 110 007, India
[c] Department of Chemistry, University of Massachusetts Lowell, MA 01854, USA

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ABSTRACT

Our earlier investigations culminated in the discovery of a unique membrane-bound enzyme Calreticulin transacetylase (CRTAase) in mammalian cells catalyzing the transfer of acetyl group from polyphenolic acetates (PAs) to certain functional proteins *viz.* Glutathione *S*-transferase (GST), NADPH Cytochrome *c* reductase and Nitric oxide synthase (NOS) resulting in the modulation of their biological activities. In order to develop SAR study, herein, we studied the influence of alkyl group at C-3 position of acetoxy coumarins on the CRTAase activity. The alkylated acetoxy coumarins lead to inhibition of catalytic activity of GST, and ADP induced platelet aggregation by the way of activation of platelet Nitric oxide synthase (NOS). Furthermore, the increase in size of the coumarin C-3 alkyl group was found to decrease the CRTAase activity.

1. Introduction

Proteins are versatile molecules for catalyst design that play a major role to sustain life processes. Modifications of proteins (enzymes) result in perturbation of their natural catalytic activity. There are two broad categories of protein modifications: (i) enzyme catalyzed covalent modifications whereby covalent electrophilic groups *viz.* acetate, phosphate, etc. are added to the side chain residue in protein, and (ii) cleavage of peptide backbone in proteins by action of proteases or less commonly by auto catalytic cleavage. Of the two methods available, covalent modification of enzymes and proteins has been used extensively for the creation of enzymes with new catalytic activities. Most of the time, the targeted modification is accomplished by acylation of amino acid residues in proteins [1]. Herein our focus is on acetyl modification on some enzyme specific proteins such as GST and Nitric oxide synthase (NOS), whereby influencing their enzymatic behavior and properties.

GST (glutathione *S*-transferases) are multifunctional proteins with representatives in all oxygen-utilizing organisms, and among all kingdoms [2]. Mammalian GSTs are the best characterized enzymes and their major function is to act as detoxifying agents. GSTs catalyze the conjugation of the tripeptide glutathione (GSH) (γ Glu-Cys-Gly) to electrophilic centers in a wide variety of potentially harmful compounds. This leads to water soluble conjugates that can be further metabolized and eventually excreted [2].

Nitric oxide (NO) is an important regulator of a variety of physiological and pathological conditions [3]. NOS catalyzes NO biosynthesis *via* a reaction involving the conversion of L-arginine to L-citrulline [4]. The acetylation of NOS is an effective approach to activate it, thereby enhancing NO levels in human platelets. Platelets are involved in the cellular mechanisms of primary homeostasis leading to the formation of blood clots. Platelets are activated when brought into contact with agents such as collagen, thrombin, and ADP. Numerous antiplatelet agents were developed based on their ability to block the receptors responsible for platelet activation [5].

Protein modification by way of acetylation of the above mentioned enzyme proteins would further extend the range of functions performed by these enzymes. The acetylation of proteins in biological systems was known to be acetyl CoA dependent until a unique membrane bound enzyme Acetoxy Drug: protein Transacetylase (TAase) was discovered in our laboratory [6–8]. TAase was found to be involved in protein modifications as it catalyzes the transfer of acetyl groups from polyphenolic acetates (PA) to the enzyme proteins such as GST, NADPH Cytochrome *c* reductase, and NOS thereby modulating their activities [9]. Several PAs, e.g. the acetoxy derivatives of coumarins, biscoumarins, xanthones, flavones, and quinolones have been examined for their substrate specificity towards TAase mediated protein acetylation. 7,8-Diacetoxy-4-methylcoumarin (DAMC), was found to be the best substrate

* Corresponding author. Fax: +91 11 2766 7206.
E-mail address: sk.sharma90@gmail.com (S.K. Sharma).

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for TAase. It catalyzes the transfer of acetyl group from DAMC to GST and results in the acetylation of several lysine residues in its active site and subsequently to inhibition of catalytic activity of GST [10]. Later TAase was identified as Calreticulin (CR), a multifunctional Ca^{2+} binding protein in Endoplasmic Reticulum (ER) lumen and this function of CR was termed *C*alreticulin transacetylase (CRTAase) [11]. A possible mechanism for the protein acetyl transferase function based on studies on CRTAase catalyzed acetylation of the receptor protein and autoacetylation of CR has already been proposed by our group [12]. The activities of other enzymes such as liver microsomal cytochrome P-450 catalyzed mixed function oxidase (MFO), NADPH Cytochrome *c* reductase, and nitric oxide synthase (NOS) were also modulated by CRTAase mediated acetylation of these enzyme proteins using acetoxycoumarins DAMC and 7-acetoxy-4-methylcoumarin (MAMC), as the acetyl donating substrates [13].

In order to develop SAR, we have studied the factors, such as the proximity of the acetoxy group to the oxygen heteroatom, the role of carbonyl group on the benzopyran nucleus, and the effect of substituents on the coumarin nucleus in modulating the substrate specificity towards CRTAse for protein acetylation [14–19].

Herein, we have compared the specificities of C-3 alkyl diacetoxy and monoacetoxy coumarins on the activity of CRTAase and CRTAase catalyzed activation of platelet NOS and also compared the structure activity relationship (SAR) with special reference to the effect of alkyl group at the C-3 position of the pyran moiety of the polyphenolic acetates. To the best of our knowledge this is the first report wherein the effect of C-3 alkyl group of coumarin on platelet aggregation inhibition has been studied.

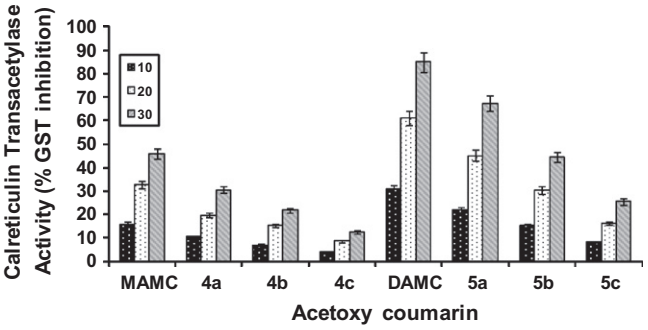


Fig. 1. CRTAase catalyzed inhibition of GST by acetoxycoumarins. Acetoxy coumarins were separately preincubated with rat liver microsomes (25 μg) and rat liver cytosol (12.5 μg) followed by assay of GST, the activity was expressed in terms of inhibition of GST. Each histogram indicates % inhibition of GST at 10 min intervals (10–30 min) in succession. Values are mean ± SD of three observations.

2. Results

In our efforts to target protein modifications *via* acetylation, a number of PAs, e.g. coumarins, biscoumarins, flavones, quinolones, xanthones, etc. were studied. Among these, the acetoxy coumarins were found to be most potent substrates for CRTAase mediated acetylation. The superior substrate specificity of coumarins further prompted us to synthesize newer analogs of this class. As an extension of our previous work on 3-alkyl-4-methylcoumarins, herein we have synthesized newer acetoxy analogs and compared their substrate specificity for CRTAase [20]. All the compounds were fully characterized on the basis of their physical and spectral data, and of the total 15 compounds synthesized, nine, i.e. **2a**, **2c**, **3b**, **3c**, **4b, 4c, 5a, 5b,** and 5c are novel. Though the remaining six compounds (**1a**–**1c** and **2b**, **3a** and **4a**) are known in literature, however, their complete spectral data is not given. Herein, we have reported the spectral data for all the compounds in the experimental section.

Due to their hydrophobic nature; the alkyl group may enhance hydrophobic interactions between the substrate (acetoxycoumarins) and the target protein, thus their effect on the rate of catalytic activity of CRTAase and the efficacy of these coumarins to activate platelet NOS was examined. We compared the specificities of both diacetoxy and monoacetoxy coumarins on CRTAase activity. It has been observed that the CRTase mediated transfer of acetyl group from diacetoxycoumarins **5a**, **5b** and **5c** when compared with DAMC showed a gradual decline with the increase in chain length of the alkyl substituent at the C-3 position in the coumarin moiety. A similar trend was observed with 7-acetoxy-4-methyl-3-alkylcoumarins **4a**, **4b** and **4c**. From the results we concluded that the specificity of polyphenolic acetates for TAase of 3-alkyl diacetoxy coumarins are almost twice as that of 3-alkyl monoacetoxy coumarins (Fig. 1). However, the TAase activities of both the mono/diacetoxy 3-alkyl coumarins are lower than the corresponding non alkylated analogs, i.e. MAMC and DAMC.

Besides the CRTase catalyzed acetylation of GST, we also studied the influence of acetoxy coumarins (**4a**–**c**, **5a**–**c**) towards activation of Nitric oxide synthase (NOS) by transferring their acetyl group to NOS. The platelets preincubated with acetoxy coumarins (**4a**–**5c**) and L-arginine exhibited enhancement in the level of NO only with compounds **4a**, **5a** and **5b**. The diacetoxy coumarins **5a** and **5b** produced more enhancements of NO levels in platelets than monoacetoxy coumarin **4a** (Fig. 2).

It has also been noticed that the acetoxy coumarins inhibit the platelet aggregation, diacetoxy coumarin **5a** having propyl group at C-3 position is much superior in exhibiting the inhibition of ADP induced platelet aggregation as compared to the corresponding monoacetoxy analog **4a**. The compounds **4b**, **4c**, **5b**, and **5c** having longer alkyl group, i.e. *n*-heptyl and *n*-nonyl at C-3 position do not inhibit platelet aggregation (Fig. 3). Thus we may conclude that

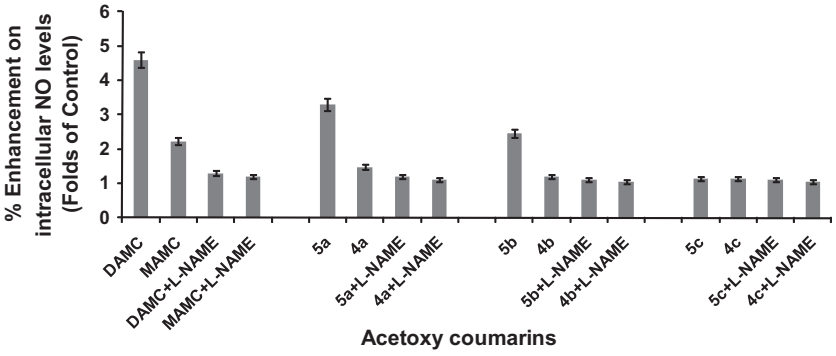


Fig. 2. The effect of C-3 substituted acetoxy coumarins on activation of platelet NOS. Acetoxy coumarins were incubated with platelets, L-arginine and DCF at 37 °C for 30 min. L-NAME was also added along with coumarins to confirm the appearance of NO. Values are mean ± SD of three observations.

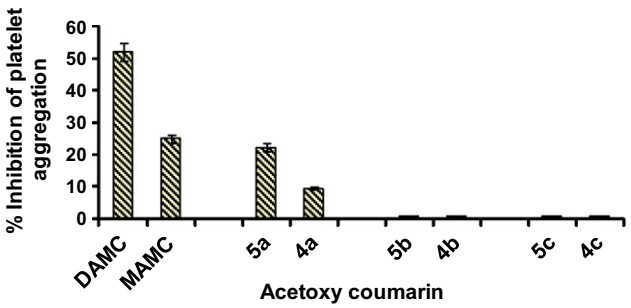


Fig. 3. The effect of C-3 substituted coumarins on platelet aggregation. PRP was incubated with acetoxy coumarin (100 μM) for 10 min at 37 °C followed by the addition of ADP (15 μM) and platelet aggregation was monitored by aggregometry. Values are mean ± SEM of three observations.

compounds with shorter alkyl group are better substrates for the inhibition of platelet aggregation. Among C-3 unsubstituted acetoxy coumarins, we found that DAMC causes highest inhibition in platelet aggregation followed by MAMC.

3. Discussion

As a part of our ongoing research program to carry out protein modification by virtue of acetylation of its amino acid residues, a wide variety of PAs as substrates for CRTAase were studied. A SAR study suggests that the presence of acetoxy groups on the benzenoid ring and the carbonyl group on the pyran nucleus are absolute requirement for CRTAase activity [13]. Herein we have studied the influence of replacing the C-3 hydrogen of coumarin with an alkyl group on the catalytic activity of CRTAase. For this we have synthesized a number of acetoxy coumarins and diacetoxy coumarins bearing different alkyl (propyl, heptyl, nonyl) substituents and then their specificities to CRTAase were determined. It was observed that the presence of bulky alkyl groups at C-3 position in the pyran ring of coumarins reduces its ability for the transfer of acetyl group to the functional proteins, suggesting that bulkier alkyl groups might be causing steric hindrance for the acetoxy substrates from accessing the active site of CRTAase. This observation was made on the basis that the compounds with propyl group at the C-3 position yielded higher CRTAase activities (Fig. 1) in comparison to their heptyl or nonyl analogs. However, the highest catalytic activity was observed with C-3 unsubstituted acetoxy coumarins MAMC and DAMC. From these results we can conclude that substitution at C-3 position by (C_3/C_7/C_9) alkyl groups led to gradual decrease in CRTAase activity.

The effect of alkyl substituents on CRTAase catalyzed acetylation of NOS was also examined. This proposition was examined using human platelets as the experimental system. The platelets preincubated with 3-substituted acetoxy coumarins and L-arginine exhibited enhancement in the level of NO, however the inclusion of L-NAME, a well-known inhibitor of NOS successfully reduce DCF fluorescence (a measure of NO level) to the control level. Thus inferring that the prior increase in DCF fluorescence without the inclusion of L-NAME was due to the increase in production of NO radicals alone (Fig. 2).

We studied the inhibition of platelet aggregation among the monoacetoxy and diacetoxy coumarins (**4a–c**, **5a–c**) and found that the results were not very promising as only two compounds, i.e. **4a** and **5a** inhibit platelet aggregation while the rest of them failed to produce any observable result (Fig. 3).

4. Conclusion

The potency of monoacetoxy and diacetoxy coumarins (**4a**, **4b**, **4c**, **5a**, **5b** and **5c**) were evaluated for the CRTAase catalyzed modulation of enzyme activity of proteins, such as inhibition of cystolic Glutathione-*S*-transferase (GST), enhancements of NO levels in platelets, and inhibition of ADP dependent platelet aggregation. It was observed that the potency of 3-alkyl 7,8-diacetoxy coumarins is almost twice as that of monoacetoxy counterparts. It can also be inferred that with the increase in chain length at C-3 position, the CRTAase activity decreases. However the C-3 unsubstituted coumarins, *viz*. MAMC and DAMC have the maximum substrate specificity for CRTAase.

5. Experimental

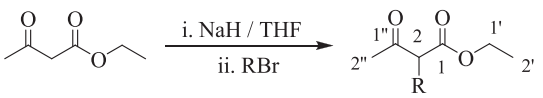
5.1. Chemistry

5.1.1. Materials

The solvents were dried and distilled prior to their use. Reactions were monitored by precoated TLC plates (Merck Silica Gel 60_{F254}); the spots were visualized either by UV light, or by spraying with 5% alcoholic $FeCl_3$ solution. Silica gel (100–200 mesh) was used for column chromatography. Sodium hydride (60% dispersed in mineral oil) was supplied from Spectrochem. Pvt. Ltd., India, and washed each time with petroleum ether. Melting points were recorded in capillaries in sulfuric acid bath and are uncorrected. UV data was recorded on Shimadzu UV-250 IPL UV–VIS spectrophotometer. Infrared spectra were recorded on Perkin–Elmer FT-IR model 9 spectrophotometer. The ^1H and ^{13}C NMR spectra were recorded on Bruker AC-400 (400 MHz, 100.6 MHz), Avance-300 (300 MHz, 75.5 MHz), and Delta Jeol-400 (400 MHz, 100.6 MHz) spectrometer using TMS as internal standard. The chemical shift values are on δ scale and the coupling constant values (J) are in hertz. The EI/HR mass spectra were recorded on Waters LCT Micromass-KC455. NADPH, Cytochrome c, reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), dichloro fluorescein–diacetate (DCFH–DA), *N*-nitro L-arginine methyl ester (L-NAME) and L-arginine were purchased from Aldrich Chemical Co., St. Louis, MO (USA). Sodium nitrite was purchased from Thomas Baker Chemicals Ltd. Mumbai, India.

5.1.2. Preparation of ethyl 2-acetylpentanoate (**1a**), ethyl 2-acetylnonanoate (**1b**) and ethyl 2-acetylundecanoate (**1c**)

To a cold solution (0 °C) of ethyl acetoacetate (10 g, 76.68 mmol) in THF (30 mL) was added sodium hydride (3.13 g, 130.36 mmol). Once the addition was complete, the reaction mixture was heated at 40 °C for 2 h. The contents of the flask were then allowed to attain room temperature, further, addition of 1-bromoalkane (1.2 eq) i.e. C_3H_7Br/$C_7H_{15}Br$/$C_9H_{19}Br$ dissolved in THF was carried out under ice cold conditions. The resultant mixture thus obtained was heated at 80 °C for 10–12 h. The reaction was monitored by TLC (10% ethyl acetate–petroleum ether). Unreacted sodium hydride was deactivated by addition of ethylacetate, the solution was then filtered and the solution washed with water, the organic layer was dried over anhydrous sodium sulfate and the solvent evaporated. The crude product so obtained was subjected to column chromatography using silica gel (100–200 mesh)



1a = *n*-C_3H_7

1b = *n*-C_7H_{15}

1c = *n*-C_9H_{19}

Scheme 1. Alkylation of ethyl acetoacetate.

and the desired product eluted with ethyl acetate–petroleum ether as viscous oil (Scheme 1).

*5.1.2.1. Ethyl 2-acetylpentanoate (**1a**).* It was obtained as yellow oil (50 %); bp: 224–225 °C (literature bp: 224–225 °C) [21]; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.93 (t, 3H, J = 7.2 Hz, H-5), 1.25–1.27 (m, 5H, H-4 and H-2′), 1.81 (m, 2H, H-3), 2.22 (s, 3H, H-2″), 3.43 (t, 1H, J = 7.3 Hz, H-2), 4.19 (q, 2H, J = 6.9 Hz, H-1′); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 13.40 (C-5), 13.70 (C-2′), 22.79 (C-4), 28.34 and 29.84 (C-3 and C-2″), 59.24 (C-2), 60.81 (C-1′), 169.53 (C-1), 202.80 (C-1″); IR (KBr) ν_{max}: 2963.57, 2937.60, 2875.95, 1741.74 (COO), 1717.86 (CO), 1466.11, 1359.67, 1188.81, 1061.86, 855.23, 753.61 cm^{-1}; HRMS: Calcd for $C_9H_{16}O_3$ $[M+H]^+$ = 173.1099. Found: 173.1120.

*5.1.2.2. Ethyl 2-acetylnonanoate (**1b**).* It was obtained as a yellow oil (53 %); bp: 266–267 °C (literature bp: 266–267 °C) [22]; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.87 (t, 3H, J = 7.0 Hz, H-9), 1.09–1.27 (m, 13H, H-4–H-8 and H-2′), 1.83 (*brm*, 2H, H-3), 2.21(s, 3H, H-2″), 3.39 (t, 1H, J = 6.7 Hz, H-2), 4.19 (q, 2H, J = 7.01 Hz, H-1′); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 13.62 (C-9), 18.06 (C-2′), 22.20 (C-8), 23.44, 25.56, 27.76, 28.62, 29.65, 32.47 (C-3–C-7 and C-2″), 59.42 (C-2), 60.68 (C-1′), 171.47 (C-1), 202.46 (C-1″); IR (KBr)ν_{max}: 2950.90, 2928.06, 2856.98, 1742.23 (COO), 1718.60 (CO), 1459.08, 1241.97, 1152.29, 1025.54, 858.30, 721.40 cm^{-1}; HRMS: Calcd for $C_{13}H_{24}O_3$ $[M+H]^+$ = 229.1725. Found: 229.1689.

*5.1.2.3. Ethyl 2-acetylundecanoate (**1c**).* It was obtained as a yellow oil (55%); bp: 292–293 °C (literature bp: 292–293 °C) [23]; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.96 (t, 3H, J = 6.9 Hz, H-11), 1.36–1.51 (m, 17H, H-4–H-10 and H-2′), 1.95 (m, 2H, H-3), 2.30 (s, 3H, H-2″), 3.48 (t, 1H, J = 6.7 Hz, H-2), 4.27 (q, 2H, J = 7.2 Hz, H-1′); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 13.62 (C-11), 19.59 (C-2′), 22.56 (C-10), 23.65, 27.29, 28.09, 28.69, 29.30, 29.61, 31.76, 32.76 (C-3–C-9 and C-2″), 59.80 (C-2), 61.04 (C-1′), 169.75 (C-1), 202.98 (C-1″); IR (KBr)ν_{max}: 2926.33, 2956.80, 2855.82, 1742.53 (COO), 1719.21 (CO) 1465.13, 1244.13, 1151.81, 1025.93, 860.17, 722.07, 646.00 cm^{-1}; HRMS: Calcd for $C_{15}H_{28}O_3$ $[M+H]^+$ = 257.3810. Found: 257.2038.

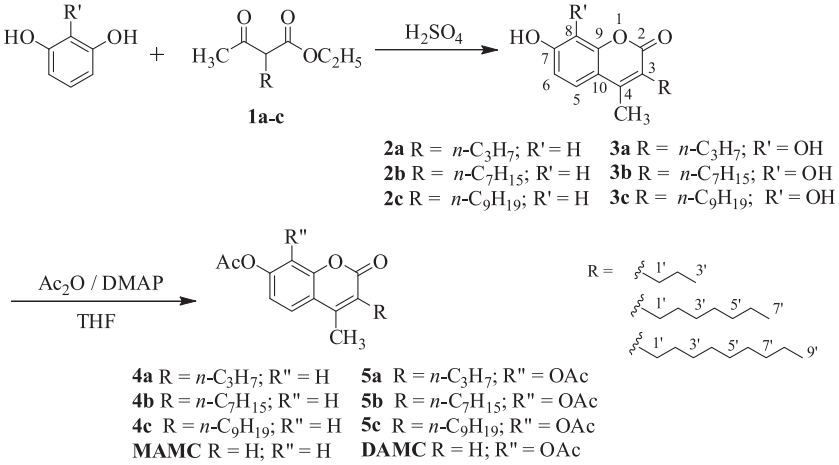
*5.1.3. General procedure for the synthesis of hydroxy coumarins (**2a–c** and **3a–c**)*

To a cold (0 °C) mixture of resorcinol/pyragallol (1 g) in alkylated ethyl acetoacetate (1.2 eq) was slowly added concentrated sulfuric acid (5 mL, dropwise). The mixture was stirred at room temperature for 3–4 h. The progress of reaction was monitored on TLC (methanol–chloroform 1:19). On completion of the reaction, ice cold water (100 mL) was added. The crude product so obtained was then filtered, washed with water, dried and crystallized from ethanol to give the monohydroxy (**2a–c**) /dihydroxy coumarins (**3a–c**) (Scheme 2).

*5.1.3.1. 7-Hydroxy-4-methyl-3-propyl-coumarin (**2a**).* It was obtained as pale yellow solid (76%); mp: 169–171 °C (literature mp: 169–171 °C) [24]; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 0.84 (t, 3H, J = 6.7 Hz, H-3′), 1.36–1.41 (m, 2H, H-2′), 2.28 (s, 3H, C-4 CH_3), 2.43 (t, 2H, J = 7.4 Hz, H-1′), 6.63 (s, 1H, H-8), 6.72 (d, 1H, J = 9.2 Hz, H-6), 7.52 (d, 1H, J = 6.8 Hz, H-5); ^{13}C NMR (DMSO-d_6, 100.6 MHz): δ 14.26 (C-3′), 15.03 (C-4 CH_3), 22.03 (C-2′), 29.11 (C-1′), 102.26 (C-8), 112.97 and 113.24 (C-6 and C-10), 121.72 (C-3), 126.88 (C-5), 147.59 (C-4), 153.61 and 160.49 (C-7 and C-9), 161.60 (C-2); IR (KBr) ν_{max}: 3442.52 (OH), 1736.68 (CO), 1713.63, 1677.15, 1606.44, 1555.38, 1475.22, 1356.83, 1303.11, 1266.92, 1243.80, 1207.49, 1146.54, 1063.11, 1008.00 880.11, 820.74, 802.57, 741.98, 665.60, 603.97, 435.26 cm^{-1}; UV (methanol) λ_{max}: 323 nm; HRMS: Calcd for $C_{13}H_{14}O_3$ $[M]^+$ = 218.0943. Found: 218.0099.

*5.1.3.2. 3-Heptyl-7-hydroxy-4-methylcoumarin (**2b**).* It was obtained as pale yellow solid (78%); mp: 63–65 °C ; ^{1}H NMR (DMSO-d_6 300 MHz): δ 0.87 (t, 3H, J = 6.4 Hz, H-7′), 1.28–1.50 (m, 10H, H-2′–H-6′), 2.40 (s, 3H, C-4 CH_3), 2.59 (t, 2H, J = 7.5 Hz, H-1′), 6.70 (d, 1H, J = 2.1 Hz, H-8), 6.82 (dd, 1H, J = 2.4 and 8.7 Hz, H-6), 7.59 (d, 1H, J = 9.0 Hz, H-5); ^{13}C NMR (DMSO-d_6, 75.5 MHz): δ 14.42 (C-7′), 15.02 (C-4 CH_3), 22.56, 27.20, 28.76, 29.03, 29.42, 31.72 (C-1′–C-6′), 102.29 (C-8), 113.00 and 113.20 (C-6 and C-10), 121.97 (C-3), 126.89 (C-5), 147.34 (C-4), 153.65 and 160.51 (C-7 and C-9), 161.52 (C-2); IR (KBr) ν_{max}: 3307.14 (OH), 1713.07 (CO), 1681.12, 1576.78, 1451.86, 1379.87, 1229.95, 1095.63, 862.94, 777.94, 532.00 cm^{-1}; UV (methanol) λ_{max}: 274 and 324 nm; HRMS: Calcd for $C_{17}H_{22}O_3$ $[M+H]^+$ = 275.3548. Found: 275.6815.

*5.1.3.3. 7-Hydroxy-4-methyl-3-nonylcoumarin (**2c**).* It was obtained as pale yellow solid (79%); mp: 46–48 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 0.86 (t, 3H, J = 6.6, H-9′), 1.27–1.50 (m, 14H, H-2′–H-8′), 2.40 (s, 3H C-4 CH_3), 2.59 (t, 2H, J = 7.6 Hz, H-1′), 6.70 (d, 1H, J = 2.4 Hz, H-8), 6.82 (dd, 1H, J = 2.4 and 8.7 Hz, H-6), 7.59 (d, 1H, J = 8.7 Hz, H-5); ^{13}C NMR (DMSO-d_6, 75.5 MHz): δ 14.40 (C-9′), 15.00 (C-4 CH_3), 22.55, 27.19, 28.43, 29.78, 29.16, 29.41, 29.43, 31.74 (C-1′–C-8′), 102.29 (C-8), 113.00 and 113.21 (C-6 and C-10), 121.99 (C-3), 126.89 (C-5), 147.34 (C-4), 153.65, 160.52



Scheme 2. Synthesis of acetoxy coumarins.

and 161.52 (C-2, C-7 and C-9); IR (KBr) ν_{max}: 3244.73 (OH), 1678.55 (CO), 1615.35, 1567.51, 1466.84, 1355.63, 1235.59, 1091.37, 851.53, 781.53, 531.91 cm^{-1}; UV (methanol) λ_{max}: 324 nm; HRMS: Calcd for $C_{19}H_{26}O_3$ $[M+H]^+$ = 303.4079. Found: 303.3478.

*5.1.3.4. 7,8-Dihydroxy-4-methyl-3-propylcoumarin (**3a**).* It was obtained as white solid (78%); mp: 189–190 °C (literature mp: 189–190 °C) [25];^{1}H NMR (DMSO-d_6, 400 MHz): δ 0.87 (t, 3H, J = 7.2 Hz, H-3′), 1.38–1.43 (m, 2H, H-2′), 2.29 (s, 3H C-4 CH_3), 2.47 (t, 2H, J = 7.2 Hz, H-1′), 6.75 (d, 1H, J = 8.4 Hz, H-6), 7.04 (d, 1H, J = 8.8 Hz, H-5), 9.19 (s, 1H, OH) and 9.87 (s, 1H, OH); ^{13}C NMR (DMSO-d_6, 100.6 MHz): δ 14.32 (C-3′), 15.19 (C-4 CH_3), 22.08 (C-2′), 29.20 (C-1′), 112.50 and 115.74 (C-6 and C-10), 113.75 (C-5), 121.59 (C-3), 132.30 (C-4), 142.37, 147.92 and 148.71 (C-7, C-8 and C-9), 161.47 (C-2); IR (KBr) ν_{max}: 3422.80 (OH), 1674.69 (CO), 1614.30, 1582.81, 1509.59, 1465.08, 1380.59, 1357.63, 1308.54, 1247.74, 1122.09, 1086.84, 1054.53, 955.99, 815.40, 774.44, 671.53, 526.46 cm^{-1}; UV (methanol) λ_{max}: 262 and 323 nm; HRMS: Calcd for $C_{13}H_{14}O_4$ $[M+H]^+$ = 235.2479. Found: 235.2624.

*5.1.3.5. 7,8-Dihydroxy-3-heptyl-4-methylcoumarin (**3b**).* It was obtained as white solid (76%); mp: 140–142 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 0.87 (t, 3H, J = 6.5 Hz, H-7′), 1.11–1.50 (m, 10H, H-2′–H-6′), 2.26 (s, 3H C-4 CH_3), 2.59 (t, 2H, J = 7.6 Hz, H-1′), 6.84 (d, 1H, J = 8.7 Hz, H-6), 7.12 (d, 1H, J = 8.7 Hz, H-5); ^{13}C NMR (DMSO-d_6, 75.5 MHz): δ 14.44 (C-7′), 15.15 (C-4, CH_3), 22.56, 27.25, 28.80, 29.05, 29.48, 31.74 (C-1′–C-6′), 112.50 and 115.72 (C-6 and C-10), 113.76 (C-5), 121.81 (C-3), 132.30 (C-4), 142.36, 147.75 and 148.70 (C-7, C-8 and C-9), 161.45 (C-2); IR (KBr) ν_{max}: 3431.39 (OH), 1673.17 (CO), 1582.51, 1510.00, 1464.89, 1380.15, 1217.35, 1124.10, 1090.72, 812.20, 777.46, 529.83, 487.46 cm^{-1}; UV (methanol) λ_{max}: 262 and 360 nm; HRMS: Calcd for $C_{17}H_{22}O_4$ $[M+H]^+$ = 291.3542. Found: 291.7042.

*5.1.3.6. 7,8-Dihydroxy-4-methyl-3-nonylcoumarin (**3c**).* It was obtained as white solid (75%); mp: 100–102 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 0.85 (t, 3H, J = 6.6 Hz, H-9′), 1.27–1.50 (m, 14H, H-2′–H-8′), 2.39 (s, 3H C-4 CH_3), 2.59 (t, 2H, J = 7.6 Hz, H-1′), 6.84 (d, 1H, J = 8.7 Hz, H-6), 7.12 (d, 1H, J = 8.7 Hz, H-5); ^{13}C NMR (DMSO-d_6, 75.5 MHz): δ 14.40 (C-9′), 15.12 (C-4 CH_3), 22.55, 27.24, 28.34, 28.76, 29.17, 29.38, 29.43, 31.75 (C-1′–C-8′), 112.50 and 115.67 (C-6 and C-10), 113.76 (C-5), 121.81 (C-3), 132.31 (C-4), 142.36, 147.69 and 148.70 (C-7, C-8 and C-9), 161.44 (C-2); IR (KBr) ν_{max}: 3421.75 (OH), 1673.01 (CO), 1582.06, 1465.42, 1309.81, 1217.12, 1122.33, 1092.80, 954.27, 811.77, 776.02, 525.55 cm^{-1}; UV (methanol) λ_{max}: 262 and 323 nm; HRMS: Calcd for $C_{19}H_{26}O_4$ $[M+H]^+$ = 319.4073. Found: 319.4775.

*5.1.4. General procedure for synthesis of 3-alkyl-7-acetoxy-4-methylcoumarin (**4a–c**)/7,8-diacetoxy-3-alkyl-4-methylcoumarin (**5a–c**)*

To a mixture of 3-alkyl-7-hydroxy-4-methylcoumarin (**2a–c**)/7,8-dihydroxy-3-alkyl-4-methylcoumarin (**3a–c**) (1 g) and dimethylaminopyridine (20 mg) in THF (20 mL) was added acetic anhydride (1.2/2.4 eq) and the reaction mixture was stirred at room temperature for 24 h. On completion of the reaction, ice cold water (100 mL) was added. The crude solid was then filtered, washed with water, dried and crystallized from ethanol to give colorless crystals of 7-acetoxycoumarins (**4a–c**)/7,8-diacetoxycoumarins (**5a–c**) (Scheme 2).

*5.1.4.1. 7-Acetoxy-4-methyl-3-propylcoumarin (**4a**).* It was obtained as white solid (72%); mp: 119–120 °C (literature mp: 119–120 °C) [24]; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.97 (t, 3H, J = 7.4 Hz, H-3′), 1.50–1.59 (m, 2H, H-2′), 2.31 (s, 3H, C-4 CH_3), 2.39 (s, 3H, –OCOCH_3), 2.62 (t, 2H, J = 7.8 Hz, H-1′), 7.01–7.05 (m, 2H, H-6 and H-8), 7.58 (d, 1H, J = 8.8 Hz, H-5); ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 14.09 (C-3′), 15.01 (C-4 CH_3), 21.14 (–OCOCH_3), 22.00 (C-2′), 25.45 (C-1′), 110.03 (C-8), 117.87 and 118.63 (C-6 and C-10), 125.28 (C-3), 126.09 (C-5), 145.52 (C-4), 151.95 and 152.63 (C-7 and C-9), 161.49 and 168.95 (C-2 and –OCOCH$_3$); IR (KBr) ν_{max}: 1765.03 (OCO), 1716.32 (CO), 1611.68, 1572.19, 1421.64, 1364.82, 1275.29, 1136.25, 1015.99, 909.45, 869.71, 776.87, 647.39, 585.88, 455.48 cm^{-1}; UV (methanol) λ_{max}: 274 and 312 nm; HRMS: Calcd for $C_{15}H_{16}O_4$ $[M+H]^+$ = 261.3016. Found: 261.3524.

*5.1.4.2. 7-Acetoxy-3-heptyl-4-methylcoumarin (**4b**).* It was obtained as white solid (73 %); mp: 58–60 °C; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.87 (t, 3H, J = 6.45 Hz, H-7′), 1.26–1.52 (m, 10H, H-2′–H-6′), 2.33 (s, 3H, C-4 CH_3), 2.40 (s, 3H, –OCOCH_3), 2.65 (t, 2H, J = 7.6 Hz, H-1′), 7.02–7.07 (m, 2H, H-6 and H-8), 7.59 (d, 1H, J = 8.4 Hz, H-5); ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ δ 14.14 (C-7′), 14.97 (C-4 CH_3), 21.15 (–OCOCH_3), 22.68, 25.62, 27.73, 28.76, 29.72 and 31.89 (C-1′–C-6′), 110.04 (C-8), 117.86 and 118.66 (C-6 and C-10), 125.23 (C-3), 126.57 (C-5), 145.28 (C-4), 151.34 and 151.91 (C-7 and C-9), 162.74 and 168.96 (C-2 and –OCOCH$_3$); IR (KBr) ν_{max}: 1770.06 (OCO), 1712.01 (CO), 1614.72, 1572.12, 1425.88, 1367.57, 1202.95, 1137.49, 1013.38, 904.82, 824.80, 777.78, 641.92 cm^{-1}; UV (methanol) λ_{max}: 275 and 360 nm; HRMS: Calcd for $C_{19}H_{24}O_4$ $[M+H]^+$ = 317.1675. Found: 317.1719.

*5.1.4.3. 7-Acetoxy-4-methyl-3-nonyl coumarin (**4c**).* It was obtained as white solid (72 %); mp: 40–42 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): 0.87 (t, 3H, J = 6.6 Hz, H-9′), 1.28–1.52 (m, 14H, H-2′–H-8′), 2.33 (s, 3H, C-4 CH_3), 2.40 (s, 3H, –OCOCH_3), 2.65 (t, 2H, J = 7.5 Hz, H-1′), 7.03–7.07 (m, 2H, H-6 and H-8), 7.59 (d, 1H, J = 8.4 Hz, H-5); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 14.33 (C-9′), 14.97 (C-4 CH_3), 21.15 (–OCOCH_3) 22.17, 27.17, 27.73, 28.77, 29.19, 29.68, 31.85 and 36.27 (C-1′–C-8′), 110.04 (C-8), 117.86 and 118.66 (C-6 and C-10), 125.23 (C-3), 126.57 (C-5), 145.28 (C-4), 151.91, 152.63 (C-7 and C-9), 161.48 and 168.96 (C-2 and –OCOCH$_3$). IR (KBr) ν_{max}: 1761.78 (OCO), 1710.66 (CO), 1616.39, 1571.48, 1427.79, 1365.89, 1208.70, 1135.54, 1014.95, 903.62, 777.04, 642.03 cm^{-1}; UV (methanol) λ_{max}: 273 and 314 nm; HRMS: Calcd for $C_{21}H_{28}O_4$ $[M]^+$ = 344.4446. Found: 344.4958.

*5.1.4.4. 7,8-Diacetoxy-4-methyl-3-propyl coumarin (**5a**).* It was obtained as white solid (78%); mp: 108–110 °C; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.97 (t, 3H, J = 7.4 Hz, H-3′), 1.50–1.58 (m, 2H, H-2′), 2.31 (s, 3H, C-4 CH_3), 2.39 (s, 6H, 2× –OCOCH_3), 2.61 (t, 2H, J = 8.0 Hz, H-1′), 7.10 (d, 1H, J = 9.2 Hz, H-6), 7.46 (d, 1H, J = 8.8 Hz, H-5); ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 14.08 (C-3′), 15.13 (C-4 CH_3), 20.38, 20.69 and 21.94 (2× –OCOCH_3 and C-2′), 29.65 (C-1′), 118.25 and 121.62 (C-6 and C-10), 119.71 (C-5), 124.91 (C-3), 130.02 (C-8), 144.06, 145.27 and 145.44 (C-4, C-7 and C-9), 160.27 (C-2), 167.61 and 167.99 (2× –OCOCH$_3$); IR (KBr) ν_{max}: 1744.14 (OCO), 1680.29 (CO), 1610.91, 1567.15, 1421.51, 1372.85, 1207.22, 1164.61, 1040.58, 988.91, 801.73, 769.75, 645.65 cm^{-1}; UV (methanol) λ_{max}: 277 and 375 nm; HRMS: Calcd for $C_{17}H_{18}O_6$ $[M]^+$ = 318.3377. Found: 318.2705.

*5.1.4.5. 7,8-Diacetoxy-3-heptyl-4-methylcoumarin (**5b**).* It was obtained as white solid (74%); mp: 68–70 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.88 (t, 3H, J = 6.6 Hz, H-7′), 1.28–1.57 (m, 10H, H-2′–H-6′), 2.33 (s, 3H, C-4 CH_3), 2.40 (s, 6H, 2× –OCOCH_3), 2.64 (t, 2H, J = 7.6 Hz, H-1′), 7.12 (d, 1H, J = 9.0 Hz, H-6), 7.48 (d, 1H, J = 9.0 Hz, H-5); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 14.12 (C-7′), 15.05 (C-4 CH_3), 20.37 and 20.69 (2× –OCOCH$_3$), 22.65, 27.78, 28.66, 29.15, 29.62 and 31.81 (C-1′–C-6′), 118.21 and 121.52 (C-6 and

C-10), 119.84 (C-5), 127.05 (C-3), 130.04 (C-8), 144.02 and 145.36 (C-7 and C-9), 141.05 (C-4), 160.34 (C-2), 167.99 and 167.99 (2× –OCOCH$_3$); IR (Nujol) ν_{max}: 1777.60 (OCO), 1706.12 (CO), 1612.52, 1461.21, 1377.37, 1274.79, 1221.62, 1114.75, 1164.43, 1078.80, 1016.08, 952.35, 880.19, 823.50, 773.93, 723.11 cm^{-1}; UV (acetonitrile) λ_{max}: 277 and 381 nm; HRMS: Calcd for $C_{21}H_{26}O_6$ [M+H]$^+$ = 375.4275. Found: 375.5471.

*5.1.4.6. 7,8-Dicetoxy-4-methyl-3-nonylcoumarin (**5c**).* It was obtained as white solid (71%); mp: 113–115 °C; ^{1}H NMR (CDCl$_3$, 300 MHz): δ 0.86 (t, 3H, J = 8.0 Hz, H-9′), 1.25–1.57 (m, 14H, H-2′–H-8′), 2.32 (s, 3H, C-4 CH$_3$), 2.39 (s, 6H, 2× –OCOC*H*$_3$), 2.62 (t, 2H, J = 7.4 Hz, H-1′), 7.11 (d, 1H, J = 8.0 Hz, H-6), 7.47 (d, 1H, J = 10.4 Hz, H-5); ^{13}C NMR (CDCl$_3$, 75.5 MHz): δ 14.10 (C-9′), 15.05 (C-4 *C*H$_3$), 20.34 and 20.65 (2× –OCO*C*H$_3$), 22.65, 27.76, 28.64, 29.29, 29.50, 29.52, 29.65 and 31.86 (C-1′–C-8′), 118.19 and 121.52 (C-6 and C-10), 119.61 (C-5), 127.03 (C-3), 129.86 (C-8), 141.08, 145.10 and 144.21 (C-4, C-7 and C-9), 160.20 (C-2), 167.35 and 167.35 (2× –OCOCH$_3$); IR (KBr) ν_{max}: 1776.75 (OCO), 1705.76 (CO), 1613.63, 1456.42, 1370.75, 1222.05, 1115.30, 1079.70, 1019.32, 881.38, 776.21, 595.79 cm^{-1}; UV (methanol) λ_{max}: 279 and 303 nm; HRMS: Calcd for $C_{23}H_{30}O_6$ [M+H]$^+$ = 403.2042. Found: 403.2109.

5.2. Microbiology

5.2.1. CRTAase assay

The test compounds were separately mixed with platelet lysate (30 μg protein), 0.25 M phosphate buffer (pH 6.5), rGST (5 μg protein) and water added to make total volume of 0.8 mL. The contents (scaled up as per requirement) were separately preincubated at 37 °C. The aliquots (0.8 mL portion) were removed periodically into spectrophotometer cuvette containing GSH (Glutathione reduced form) and 1-chloro-2,4-dinitrobenzene (CDNB) to make their final concentration 1 mM in a total volume of 1 mL and GST was assayed. The procedure outlined above with the addition of DMSO (vehicle for test compound), served as a control. The effect of the test compound on preincubation with platelet TAase mediating the inhibition of GST was represented by plotting the inhibition of GST against time of preincubation. The extent of inhibition of GST will be considered proportional to TAase activation. The unit of TAase was expressed in terms of inhibition of GST.

5.2.2. Isolation of platelet rich plasma (PRP)

Venous blood (9 mL) was collected from healthy volunteers (n = 45; age, 27 ± 1.2 yrs) for this study after full explanation to them about the details of the experiment and taking their consent. Approval of the Ethical Committee was obtained from Vallabhbhai Patel Chest Institute, University of Delhi, Delhi, India. The citrated blood was used for the preparation of PRP by mixing with 1.0 mL of 3.8% trisodium citrate (anticoagulant). The citrated blood was centrifuged at 1200 rpm for 10 min at room temperature. The upper two-third fraction of plasma (PRP) was transferred to another centrifuge tube leaving behind lower one third layer to avoid contamination with WBC's and RBC's. PRP was then further centrifuged at 4000 rpm for 5 min to produce a platelet button. The platelet button was then suspended in PBS (phosphate buffered saline).

5.2.3. Assay of NOS by flow cytometry

The method outlined by Imrich and Kobzik was followed for the assay of NOS by flow cytometry [26]. Measurements were made with a 488 nm laser based flow cytometer (FACS calibur, Becton and Dickenson, USA) and data (light scatter and green fluorescence) was acquired using the Cell Quest software (Becton and Dickinson, USA). Analysis was performed by applying appropriate gates with reference to the auto fluorescence measured under similar conditions. PRP was incubated with polyphenolic acetates (100 μM) for 10 min and the activity was triggered by the addition of ADP (15 μM) for 5 min. Platelets were palleted down and were washed twice with PBS and further preincubated in buffer containing 1 μL of DCF-DA (dissolved in CH$_3$OH) in a total volume of 1 mL to make the final concentration 2 μM and kept at 37 °C for 30 min and then the reaction was stopped by placing the tubes containing the reaction mixture over ice. One set of PRP aliquots was preincubated with L-NAME (50 μM) for 30 min before the addition of test compounds and subjected to NOS assay.

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