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Synthesis of benzoyl phenyl benzoates as effective inhibitors for phospholipase A_2 and hyaluronidase enzymes

Shaukath Ara Khanum,[a] Satish Kumar Murari,[b] Bannikuppe Sannanaik Vishwanth[b] and Sheena Shashikanth[a,*]

[a]*Department of Studies in Chemistry, University of Mysore, Manasagangotri, Mysore 570 006, India*
[b]*Department of Studies in Biochemistry, University of Mysore, Manasagangotri, Mysore 570 006, India*

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Abstract—Benzoylation of (hydroxy phenyl) phenyl methanone **2a**–**g** to benzoyl phenyl benzoates **4a**–**g**, a benzophenone analogue, was achieved in good yield. All the newly synthesized compounds were evaluated for their phospholipase A_2 [E.C. 3.1.1.4] and hyaluronidase [E.C. 3.2.1.35] enzyme inhibitory activity in snake venom as source and their structure–activity relationship with respect to different groups is reported for the first time. The in vitro PLA_2 enzyme inhibitory activity and in vivo anti-inflammatory activity studies of benzoyl phenyl benzoates are illustrated.

1. Introduction

Phospholipase A_2 (PLA_2) is a promising family of distinct enzymes that exhibits different substrate specificities, cofactor requirements, subcellular localization and cellular functions.[1] The PLA_2 class of enzymes catalyzes hydrolysis of the 2-acyl ester of 3-Sn phosphoglycerides to yield arachidonic acid (metabolized to eicosanoids by cyclooxygenase and lipoxygenase) and lysophospholipid, which is a rate limiting step of the production of pro-inflammatory lipid mediators such as prostaglandins, leukotrienes, lipoxins, and platelet activating factor.[2–5] In many inflammatory diseases, high levels of PLA_2 enzymes are identified and are believed to be responsible for part of the inflammatory reactions. Injection of purified PLA_2 enzyme from synovial fluid and from snake venom into animal joints confirmed the development of an acute inflammatory response with edema, swelling of synovial cells, and hyperplasia.[6,7] Clinical results with cyclooxygenase and lipoxygenase inhibitions demonstrate that inhibition of PLA_2 enzyme results in reduction of both lipid mediators, indicating that these PLA_2 inhibitors can be used as anti-inflammatory drugs.

The competence of benzophenone analogues as chemotherapeutic agents, especially as anti-inflammatory compounds is well recognized.[8–13] Recently, synthesis and structure–activity relationship of benzophenones as novel class of p38 MAP kinase inhibitors with high anti-inflammatory activity has been reported.[14] Moreover, Katsuichi et al. [15] have reported benzophenone analogues as PLA_2 inhibitors. In view of these observations and due to our ongoing work on microwave technique,[16,17] this investigation is directed towards the synthesis and evaluation of benzophenone analogues as anti-inflammatory agents.

The enzyme hyaluronidase and its substrate hyaluronan are known to be involved in fundamental, physiological and pathological, processes such as embryological development, migration, adhesion, proliferation and differentiation of cells, immune surveillance, inflammation, wound healing, angiogenesis, tumorigenesis, virulency, and venom.[18] Hyaluronidases are present in virtually all snake venoms.[19] In snake venoms, the hyaluronidases are generally referred to as spreading factors.[20] The spreading property of enzyme is presumed to be the critical event in the spreading of toxins from the site of injection to systemic circulation.[21] This process is accomplished by the degradation of hyaluronan and eventual loss of integrity in extracellular matrix of soft connective tissue surrounding the blood vessels. This leads to the easy diffusion of other toxic components of venoms.

Keywords: Phospholipase A_2 inhibitor; Hyaluronidase inhibitor; Benzoyl phenyl benzoates.
* Corresponding author. Tel.: +91 082 125 47279; fax: +91 082 125 18358; e-mail: skanth1@rediffmail.com

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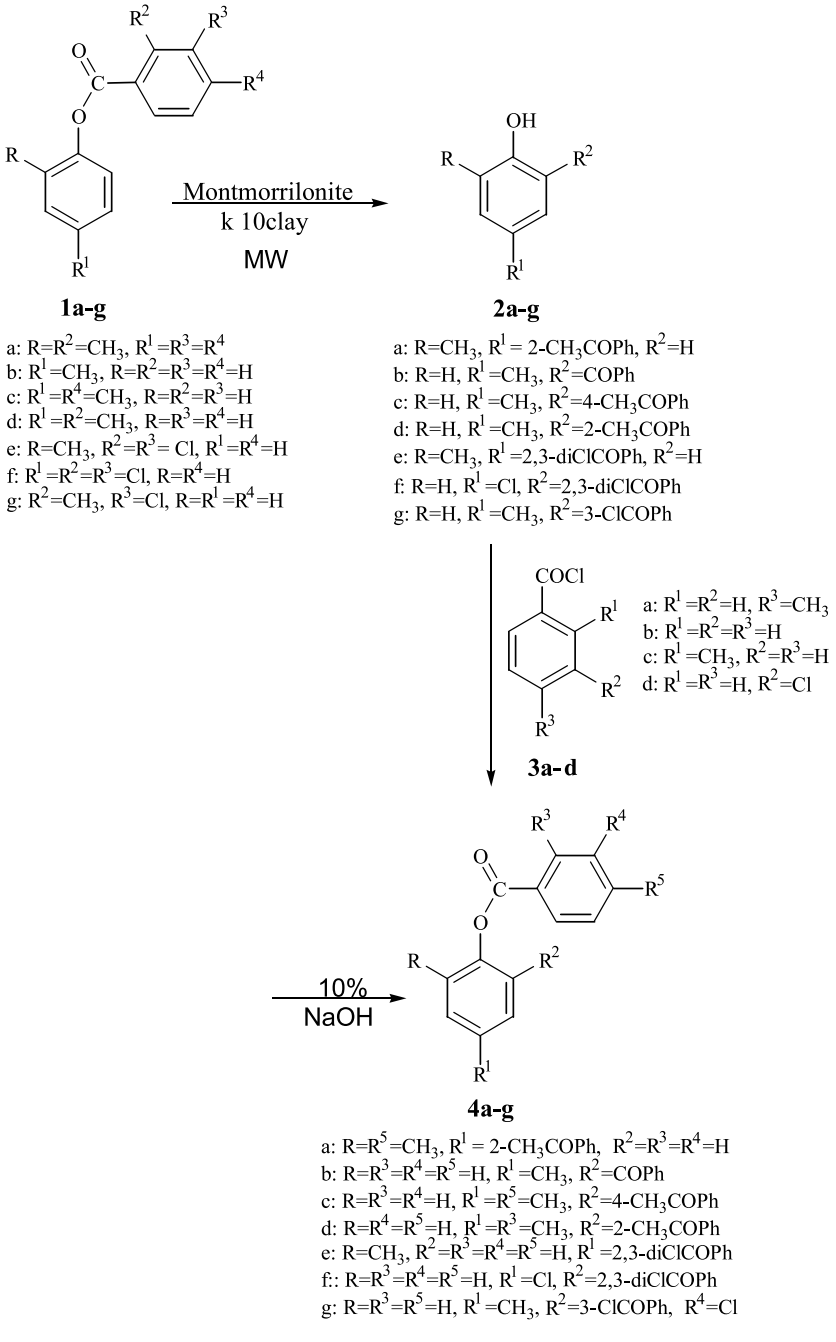
Distortion in the integrity of the extracellular matrix of local tissue(s) due to degradation of hyaluronan with an eventual dissemination of target specific toxins is presumed to be the critical event in the enzyme-mediated spreading process.[18] The infective nematodes release hyaluronidase during the gastrointestinal invasive stage and are thought to have a role in tissue degradation and mucosal invasion, as well as in the pathogenesis of the associated enteritis.[21] Certain anti-inflammatory drugs that are claimed as possessing hyaluronidase inhibitory activity include salicylates indomethacin, sodium cromoglycate, and sodium auro-thiomalate.[22] These drugs may exert a portion of their inflammatory activity by preventing the generation of small Hyaluronic acid (HA) fragments, though the fragments of HA are potent inducers of inflammatory cytokine release.[23] Other drugs that are used to suppress allergic reactions, such as disodium cromoglycate, flavonoids, tannins, curcumins, glycyrrhizin, cinnamic acid derivatives, and tranilast may function as hyaluronidase inhibitors.[24,25] Synthetic inhibitors prevent venom induced systemic coagulopathy and local tissue damage.[26] No information is so far available on the active site residues and mechanism of hyaluronan degradation by snake venom hyaluronidases.[18] Inhibition of hyaluronidase(s) contributes toward the better management of snakebites. It is likely that the cocktail of the inhibitors are of great value as first aid agents and further, incorporation would complement



Scheme 1.

and enhance the efficiency of antivenom therapy as systemically administered antivenoms have minimal protective effects on local tissue damage.[19,20] This study emphasizes the need for screening of compounds and a thorough characterization of locally acting enzymes/toxins and suggests for identification/designing of novel inhibitor(s).

2. Chemistry

The synthetic sequence is outlined in Scheme 1. Compounds **1a**–**g** on thoroughly mixing with an equal amount of montmorrillonite k 10 clay in the solid state using vortex mixer and on subjecting to microwave irradiation for 10–13 min afforded substituted (hydroxy phenyl)phenyl methanone **2a**–**g** in excellent yield.[16,17] Benzoylation of **2a**–**g** with the respective benzoyl chlorides **3a**–**d** affords substituted benzoyl phenyl benzoate **4a**–**g**.[13] The compounds **2a**–**g**[27] and **4a**–**g**[28] were characterized by IR, ^{1}H NMR, and mass spectroscopies.

3. Pharmacological evaluation

3.1. PLA_2 activity

This was assayed with [^{14}C]oleate-labeled autoclaved *Escherichia coli* as the substrate.[29] The reaction mixture, 350 μl contained 100 mM Tris–HCl, pH 8.0, 5 mM Ca^{2+}, and 3.15×10^9 autoclaved *E. coli* cells (corresponding to 10,000 cpm and 60 nmol of lipid phosphorous). The amount of enzyme protein was chosen such that 10–15% hydrolysis of substrate was obtained when incubated at 37 °C for 60 min. The reaction components were mixed in the following order: buffer, calcium, water, and benzoyl phenyl benzoates. Adding labeled *E. coli* substrate started the reaction. The reaction was terminated by adding 100 μl 2.0 M HCl and 100 μl of fatty acid free BSA (100 mg/ml). The tubes were vortex-mixed and centrifuged at 20,000*g* for 5 min. An aliquot (140 μl) of the supernatant containing released [^{14}C]oleic acid was mixed with scintillation cocktail and counted in a Hewlett Packard liquid Scintillation Analyzer TRI CARB 2100 TR.

3.2. Determination of edema inducing activity

Groups of six mice (22–24 g) were injected in the right footpads of hind limbs with 3 mM dose of benzoyl phenyl benzoates in 20 μl saline. The left footpads received 20 μl of saline, which served as control. After 45 min, the mice were sacrificed by cervical dislocation and both legs were cut at the ankle joint and weighed individually. The increase in weight due to edema was calculated as the edema ratio which equals the weight of edematous leg × 100/weight of the normal leg Minimum edema dose is defined as micrograms of protein causing an edema ration. of 120%. The time-course of the edema-inducing activity was obtained by injecting a fixed dose of protein into mice footpads and killing them at regular time periods. Edema ratio was calculated as defined.[30]

3.3. Hyaluronidase assay

Hyaluronidase activity was assayed by estimating the amount of *N*-acetylglucosamine released.[31] Snake venom 100 μg each was separately incubated with 50 μg of hyaluronic acid in 300 μl 0.2 M sodium acetate buffer, pH 5.0 containing 0.15 M NaCl and 0.88 mM 4-(2-methylbenzoyl)-2-methylphenyl 4-methylbenzoate derivatives at 37 °C for 2.5 h. The change in absorbance was monitored at 585 nm. The results are shown in Table 2.

4. Results and discussion

As shown in Table 1, among compounds **4a**–**g**, **f** and **g** demonstrated strong in vitro and in vivo PLA_2 enzyme inhibition. Compounds **4c** and **b** inhibited the in vitro PLA_2 activity of *Naja melanoleuca* venom and *Trimeresurus flavoviridis* venom isoenzymes, respectively. The edema ratio at 1 μg PLA_2 enzyme concentration dropped below 120% in the presence of **4a**–**g** at 300 μM concentration with all used PLA_2 isoenzymes. Compounds **4f** and **g** were more effective compared to

Table 1. Neutralizing edema inducing activity and IC_{50} values in μM of compounds **4a**–**g** on PLA_2 enzyme activity

Phospholipase A_2	**4a**	**4b**	**4c**	**4d**	**4e**	**4f**	**4g**	Ursolic acid
Naja melanoleuca venom	73 (ND)	60 (ND)	57 (103 ± 6)	98 (102 ± 5)	132 (ND)	53 (104 ± 1)	35 (101 ± 7)	2.9 101 ± 6
Echis carinatus venom	87 (ND)	94 (ND)	96 (ND)	105 (ND)	112 (ND)	40 (103 ± 4)	31 (101 ± 3)	2.3 101 ± 3
Trimeresurus flavoviridis venom	75 (ND)	69 (105 ± 2)	108 (ND)	139 (ND)	89 (ND)	46 (105 ± 3)	53 (109 ± 3)	2.5 102 ± 1
Synovial fluid[a]	56 (101 ± 6)	46 (109 ± 2)	123 (ND)	86 (106 ± 3)	156 (ND)	32 (102 ± 1)	34.9 (103 ± 4)	2.5 101 ± 2

Edema ratio (given in parentheses) = weight of edematous leg × 100/weight of normal leg.
ND = not determined.
The PLA_2 enzyme (1 μg): compounds **4a**–**g**/ursolic acid (300 μM) mixture was preincubated at 37 °C for 1 h prior to injection into the mice foot pads. Values of edema ratio are expressed as mean ± SD ($n = 4$), P values < 0.05 were considered significant when compared to the control by Student's t test.
[a] Amount of venom taken for each assay was 1.5 μg (600 ng).

Table 2. Effect of compounds **4a**–**g** on hyaluronidase enzyme activity

Hyaluronidase enzyme source	Percent inhibition of compounds (inhibitors)						
	4a	**4b**	**4c**	**4d**	**4e**	**4f**	**4g**
Naja naja naja	42.4	13.9	69.3	58.7	NI	85.3	89.3
Naja melanoleuca	36.7	NI	NI	46.9	16.9	90.6	79.2
Vipera russelli russelli	36.9	28.2	46	NI	26.2	71.6	87.8
Trimeresurus elegans	48.9	NI	76.8	42.4	33.1	76.8	92.5

NI = No inhibition.

Values are presented as mean of four independent experiments. Heparin was used as known inhibitor in the same concentration as compounds **4a**–**g** and it showed 100% inhibition of hyaluronidase enzyme in all mentioned snake venoms as source.

4c and **e** in both in vivo anti-inflammatory activity and in vitro PLA_2 enzyme inhibition.

Compounds **4a**–**g** alone did not cause edema when injected into mice footpads. Furthermore, it may be seen from Table 1 that compounds **4f** and **g** with a benzoyl group at the *ortho* position carrying the electron-withdrawing chloro group show higher inhibitory activity than compounds **4a**–**e**. In conclusion, it may be said that **4f** and **g** in vitro inhibitory activity correlates with its in vivo inhibition of edema induced by PLA_2 enzyme isoforms. Similar trend has been observed in **4b** and **c** with *T. flavoviridis* venom PLA_2 and *Naja melanoleuca* venom PLA_2. Synovial fluid PLA_2 inhibitory activity was higher with **4b** than compounds **4a**, **c**, and **d**. Our current efforts are directed toward improving the anti-inflammatory properties. We propose that **4f** and **g** are potential anti-inflammatory compounds.

As shown in Table 2, compounds **4a**, **f**, and **g** demonstrate inhibition toward Elapidae, Viperidae, and Crotalidae families of snake venom hyaluronidases and among them **4f** and **g** showed strong inhibition in the snake venom used as source for hyaluronidase enzyme. Compounds **4c**–**e**, showed no inhibition to *N. melanoleuca*, *Vipera russelii russelii* and *Naja naja naja*, respectively. Further **4f** and **g** were found to show good inhibition of hyaluronidases in all species of snake venoms. So far no information is available on the active site residues and mechanism of hyaluronan degradation by snake venom hyaluronidases. However X-ray crystallography studies indicate the primary catalytic residues as Asn 349, His 399 and Tyr 408. The hyaluronidase enzyme binds compounds **4f** and **g** in which *ortho*-substituted benzoyl group carries the electron-withdrawing chloro group. Therefore a higher inhibition was obtained with **4f** and **g** than with the corresponding compounds **4a**–**e**. Our current efforts are directed toward identifying and designing inhibitors for hyaluronidase enzyme.

Results from this preliminary study would be beneficial in developing anti-snake venom preparations to treat local wound effects, including hemorrhagic bullae and muscle necrosis from naja and viper bites.

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27. Compound **2a**: Yield 92%, mp 120–122 °C; IR (Nujol) ν (cm^{-1}): 1640 (C═O), 3320–3450 (OH); ^1H NMR ($CDCl_3$) δ: 2.3 (s, 6H, $2CH_3$), 6.8–7.5 (m, 7H, Ar-H), 9.7 (br s, 1H, OH); EI-MS: *m/z* (85%) M^+ 226. Anal. Calcd for $C_{15}H_{14}O_2$: C, 79.64; H, 6.19. Found: C, 79.62; H, 6.20.
Compound **2b**: Yield 91%, mp 81–83 °C; IR (Nujol) ν (cm^{-1}): 1670 (C═O), 3545–3649 (OH); ^1H NMR ($CDCl_3$) δ: 2.3 (s, 3H, CH_3), 6.85–7.75 (m, 8H, Ar-H), 12.05 (br s, 1H, OH); EI-MS: *m/z* (87%) M^+ 212. Anal. Calcd for $C_{14}H_{12}O_2$: C, 79.24; H, 5.66. Found: C, 79.26; H, 5.64.
Compound **2c**: Yield 92%, mp 75–77 °C; IR (Nujol) ν (cm^{-1}): 1665 (C═O), 3510–3641 (OH); ^1H NMR ($CDCl_3$) δ: 2.3–2.35 (2s, 6H, $2CH_3$), 6.9–7.7 (m, 7H, Ar-H), 12.1 (br s, 1H, OH); EI-MS: *m/z* (83%) M^+ 226. Anal. Calcd for $C_{15}H_{14}O_2$: C, 79.64; H, 6.19. Found: C, 79.63; H, 6.16.
Compound **2d**: Yield 90%, mp 71–73 °C; IR (Nujol) ν (cm^{-1}): 1665 (C═O), 3515–3645 (OH); ^1H NMR ($CDCl_3$) δ: 2.25–2.3 (2s, 6H, $2CH_3$), 6.9–7.7 (m, 7H, Ar-H), 12.05 (br s, 1H, OH); EI-MS: *m/z* (83%) M^+ 226. Anal. Calcd for $C_{15}H_{14}O_2$: C, 79.64; H, 6.19. Found: C, 79.62; H, 6.18.
Compound **2e**: Yield 86%, mp 154–156 °C; IR (Nujol) ν (cm^{-1}): 1645 (C═O), 3300–3410 (OH); ^1H NMR ($CDCl_3$) δ: 2.3 (s, 3H, CH_3), 6.9–7.65 (m, 6H, Ar-H), 10.1 (br s, 1H, OH); EI-MS: *m/z* (85%) M^+ 281. Anal. Calcd for $C_{14}H_{10}Cl_2O_2$ (281): C, 59.78; H, 3.55; Cl, 25.26. Found: C, 59.79; H, 3.58; Cl, 25.28.
Compound **2f**: Yield 85%, mp 78–80 °C; IR (Nujol) ν (cm^{-1}): 1648 (C═O), 3305–3410 (OH); ^1H NMR ($CDCl_3$) δ: 6.9–7.65 (m, 6H, Ar-H), 11.9 (br s, 1H, OH); EI-MS: *m/z* (84%) M^+ 301.5. Anal. Calcd for $C_{13}H_7Cl_3O_2$: C, 51.78; H, 2.34; Cl, 35.27. Found: C, 51.77; H, 2.33; Cl, 35.26. Compound **2g:** Yield 92%, mp 71–73 °C; IR (Nujol) ν(cm^{-1}): 1673 (C═O), 3550–3640 (OH); ^1H NMR ($CDCl_3$) δ: 2.2 (s, 3H, CH_3), 7.0–7.65 (m, 7H, Ar-H), 12.15 (br s, 1H, OH); EI-MS: *m/z* (88%) M^+ 246.5. Anal. Calcd for $C_{14}H_{11}ClO_2$: C, 68.15; H, 4.46; Cl, 14.40. Found: C, 68.17; H, 4.44; Cl, 14.42.
28. Compound **4a**:[13] Yield 78%, mp 53–55 °C; IR (Nujol) ν (cm^{-1}): 1750 (ester, C═O); 1665 (C═O), ^1H NMR ($CDCl_3$) δ: 2.32–2.51 (3s, 9H, $3CH_3$), 6.85–7.6 (m, 11H, Ar-H); EI-MS: *m/z* (75%) M^+ 344. Anal. Calcd for $C_{23}H_{20}O_3$: C, 80.21; H, 5.85. Found: C, 80.22; H, 5.86.
Compound **4b**:[13] Yield 75%, mp 89–91 °C; IR (Nujol) ν (cm^{-1}): 1760 (ester, C═O); 1670 (C═O), ^1H NMR ($CDCl_3$) δ: 2.2 (s, 3H, CH_3), 6.9–7.7 (m, 13H, Ar-H); EI-MS: *m/z* (76%) M^+ 316. Anal. Calcd for $C_{21}H_{16}O_3$: C, 79.73; H, 5.10. Found: C, 79.75; H, 5.12.
Compound **4c**:[13] Yield 78%, mp 97–99 °C; IR (Nujol) ν (cm^{-1}): 1750 (ester, C═O); 1668 (C═O), ^1H NMR ($CDCl_3$) δ: 2.3–2.5 (3s, 9H, $3CH_3$), 6.8–7.6 (m, 11H, Ar-H); EI-MS: *m/z* (75%) M^+ 344. Anal. Calcd for $C_{23}H_{20}O_3$: C, 80.21; H, 5.85. Found: C, 80.23; H, 5.83.
Compound **4d**:[13] Yield 79%, mp 96–98 °C; IR (Nujol) ν (cm^{-1}): 1735 (ester, C═O); 1655 (C═O), ^1H NMR ($CDCl_3$) δ: 2.3–2.5 (3s, 9H, $3CH_3$), 6.92–7.7 (m, 11H, Ar-H); EI-MS: *m/z* (74%) M^+ 344. Anal. Calcd for $C_{23}H_{20}O_3$: C, 80.21; H, 5.85. Found: C, 80.22; H, 5.84.
Compound **4e**: Yield 77%, mp 56–57 °C; IR (Nujol) ν (cm^{-1}): 1737 (ester, C═O); 1658 (C═O), ^1H NMR ($CDCl_3$) δ: 2.3 (s, 3H, CH_3), 6.9–7.65 (m, 11H, Ar-H); EI-MS: *m/z* (73.5%) M^+ 385. Anal. Calcd for $C_{21}H_{14}Cl_2O_3$: C, 65.47; H, 3.66; Cl, 18.41. Found: C, 65.46; H, 3.65; Cl, 18.42.
Compound **4f**: Yield 77%, mp 90–92 °C; IR (Nujol) ν (cm^{-1}): 1740 (ester, C═O); 1660 (C═O), ^1H NMR ($CDCl_3$) δ: 6.9–7.65 (m, 11H, Ar-H); EI-MS: *m/z* (74%) M^+ 405.5. Anal. Calcd for $C_{20}H_{11}Cl_3O_3$: C, 59.22; H, 2.73; Cl, 26.22. Found: C, 59.23; H, 2.74; Cl, 26.23.
Compound **4g**:[13] Yield 77%, mp 93–95 °C; IR (Nujol) ν (cm^{-1}): 1755 (ester, C═O); 1670 (C═O), ^1H NMR ($CDCl_3$) δ: 2.3 (s, 3H, CH_3), 6.75–7.7 (m, 11H, Ar-H); EI-MS: *m/z* (77%) M^+ 385. Anal. Calcd for $C_{21}H_{14}Cl_2O_3$: C, 65.47; H, 3.66; Cl, 18.41. Found: C, 65.45; H, 3.65; Cl, 18.40%.
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