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Synthesis and anti-inflammatory evaluation of novel C66 analogs for the treatment of LPS-induced acute lung injury

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Abstract

We previously reported a symmetric mono-carbonyl analog of curcumin (MACs), C66, which demonstrated potential anti-inflammatory activity and low toxicity. In continuation of our ongoing research, we designed and synthesized 34 asymmetric MACs based on C66 as a lead molecule. A majority of the C66 analogs effectively inhibited LPS induction of TNF-α and IL-6 expression. Additionally, a preliminary SAR was conducted. Furthermore, active compounds **4a11** and **4a16** were found to effectively reduce the W/D ratio in the lungs and the protein concentration in the bronchoalveolar lavage fluid (BALF). Meanwhile, a histopathological examination indicated that these two analogs significantly attenuate tissue injury in the lungs with LPS-induced ALI rats. **4a11** and **4a16** also inhibited mRNA expression of several inflammatory cytokines, including TNF-α, IL-6, IL-1β, COX-2, ICAM-1 and VCAM-1, in the Beas-2B cells after LPS challenge. Altogether, the data exhibits a series of new C66 analogs as promising anti-inflammatory agents for the treatment of LPS-induced ALI.

Keywords: C66; Acute lung injury; Drug design; Synthesis; Chemical stability.

1. Introduction

Clinical acute lung injury (ALI), characterized by alveolar-capillary membrane disruption, extensive leukocyte infiltration, and the release of pulmonary edema associated with proteinaceous alveolar exudates, is a life threatening syndrome that causes high morbidity and mortality worldwide (1, 2). Despite decades of research, only several clinical therapies for ALI have emerged, and the mortality of ALI still remains more than 40% (3). Abundant evidence has accumulated in recent years to indicate that inflammatory cytokines play a major role in mediating, amplifying, and perpetuating the process of ALI (4-6). Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), two well-known cytokines have been identified to express highly in the bronchoalveolar lavage fluids (BALF) in both humans and animals with ALI (7, 8). Therefore, a blockade of the proinflammatory cytokines, such as

TNF-α or IL-6, was used as a classical strategy for the development of new anti-inflammatory agents in the treatment of ALI.

Curcumin, an active ingredient of turmeric plant, appears to be useful in a wide range of pharmacological activities such as an anti-carcinogenic (9), anti-oxidant (10, 11), anti-angiogenic and anti-tumor activities (12-14). In particular, curcumin is also well-known for its anti-inflammatory properties, showing high inhibition towards the release of inflammatory cytokines thus acting as an anti-inflammatory agent in acute as well as in chronic models of inflammation (15, 16). However, the clinical application of curcumin is restricted due to its aqueous insolubility, poor bioavailability and rapid metabolism (17). It is suggested that the presence of the methylene group and β-diketone moiety causes the instability of curcumin under physiological conditions (18). To enhance the stability of curcumin, numerous approaches have been conducted, including reported mono-carbonyl analogs of curcumin (MACs) with structural modifications with the deletion of the β-diketone moiety (19, 20). Several MACs were reported to have an enhanced stability *in vitro* and an improved pharmacokinetic (PK) profile *in vivo*, therefore MACs have attracted a considerable attention for their potential bioactivities and PK properties.

Previously, we reported the synthesis and bioscreening for a series of symmetric mono-carbonyl analogues of curcumin (MACs). Amongst these analogues, (2*E*,6*E*)-2,6-bis[2-(trifluoromethyl)benzylidene] cyclohexanone (**C66**) exhibited enhanced stability and potential anti-inflammatory properties by suppressing the LPS-induced expression of TNF-α and IL-6 (21, 22). Moreover, a preliminary structure-activity relationships (SAR) study concluded that structural modifications of the 2-electronegativity in ring A of the parent molecule was not tolerated (23). Based on the SAR examination and C66's anti-inflammatory properties, we sought to develop new therapeutic agents for treatment of ALI. The present study therefore uses a series of asymmetric C66 analogs that were designed, synthesized and evaluated for their anti-inflammatory activities.

2. Results and discussion

2.1. Chemistry

The synthesis of new asymmetric C66 analogs and chemical optimizations on the aromatic ring are outlined in Scheme 1 and Table 1, respectively. The key intermediates of 2-(trifluoromethyl)benzylidene cyclopentanone **3a** and 2-(trifluoromethyl)benzylidene cyclohexanone **3b** were achieved in 2 steps from commercially available cyclopentanone (**1a**) and cyclohexanone (**1b**) as shown in Scheme 1, which are presented in detail from our previous publications (24). Briefly, cyclopentanone (**1a**) and cyclohexanone (**1b**) were exposed to morpholine in hot toluene for 4 h to give morpholine enamines **2a-b** without further purification. Aldol condensation of enamines **2a-b** and 2-(trifluoromethyl)benzaldehyde in the presence of saturate HCl provided key intermediates **3a** and **3b,** respectively. Treatment with various aromatic aldehydes, in an aqueous NaOH solution or dry HCl gas, on **3a** or **3b** was conducted to furnish the desired C66 analogs (Table1) with satisfied yields.

2.2 Inhibitory screening against LPS-induced TNF-a and IL-6 release by C66 analogs

Lipopolysaccharide (LPS), one of most powerful bacterial virulence factors in terms of proinflammatory properties, was used to initiate acute inflammatory responses in mammals that are susceptible to the host reaction of tissue injury or infection (25). Two well-known cytokines, TNF-α and IL-6, are produced mainly in cells of reticuloendothelial origin (e.g., macrophages), especially when induced by external stimuli, such as LPS. Herein, C66 and its 34 synthetic analogs were evaluated for their inhibitory abilities against LPS-induced TNF-α and IL-6 release in mouse RAW 267 macrophages. Macrophages were stimulated with LPS in the absence or presence of the test samples at a concentration of 10 μM, where the leading compound C66 showed a poetical inhibition against TNF-α and IL-6 production. The cells were pre-incubated for 30 min with the C66 analogs and C66 as a positive control. After that, cells were treated with LPS (0.5 μg/mL) for 24 h at 37 ^{o}C. The concentration of TNF-α and IL-6 in media was detected through enzyme-linked immunosorbant assay (ELISA) and normalized by the protein concentration of cells harvested in homologous culture plates.

The results of the anti-inflammatory assay of tested compounds are shown in Figure 1A and B, respectively. The results demonstrated that the majority of synthetic compounds inhibited LPS-induced TNF-α and IL-6 expression in various degrees at a dosage of 10 μM. Among these tested compounds, **4a2**, **4a5-9**, **4a11** and **4a15-17** exhibited stronger inhibitory abilities than C66 in IL-6 expression (inhibitory rates>50%, Fig. 1A). Meanwhile, multiple compounds of **4a7**, **4a11**, **4a16**, **4b4**, **4b5**, and **4b15**, showed a moderate inhibitory effect on LPS-induced TNF-α release with a wide inhibitory rate, ranging from 50% to 80% (Fig. 1B). In particular, compounds **4a11**, **4a16** and **4a17**, exhibited a stronger inhibition of both LPS-induced IL-6 and TNF-α production with their inhibitory rates reaching 82%, 87%, 56% (anti-IL-6) and 49%, 80%, 50% (anti-TNF-α), respectively, compared to the LPS control.

2.3 Structure-activity relationship analysis of C66 analogs

Our previous studies showed that symmetric MACs bearing an electron-withdrawing halogen substituent at position-2 of the A-ring had excellent anti-inflammatory activity (21, 23). Therefore, we sought to probe the role of various functional groups in the aromatic ring for 2-CF_3 in ring A substituted asymmetric C66 analogs. Combined with the current bio-screening, it is generally observed that cyclopentanone-containing analogs (series 4a) are more effective in decreasing LPS-induced IL-6 and TNF-α secretion than that of nearly inactive cyclohexanone-containing series (series 4a). Especially, the obtained compounds **4a11**, **4a16** and **4a17**, containing a methoxy or 2-(dimethylamino)propanoxy group attached at the *meta*- or *para*- position on aromatic rings, exhibited the strongest inhibitory effect. Unfortunately, when the methoxy group was replaced with alkoxyl group (**4a1**, **4a10**), halogens (**4a3-4**, **4a10**, **4a13-15**), or *ortho*-methoxy group (**4a12**), the anti-inflammatory activity was slightly decreased. Interestingly, in this series, we observed that heterocycle derivatives (**4a6**, **4a7**, **4a8**, and **4a9**) seemed to have additional anti-inflammatory activity. However, it is difficult to estimate the SAR of cyclohexanone-containing C66 analogs (series 4b) related to TNF-α and IL-6 inhibitions due to their relatively low activity. These results showed that the cyclic ketone substructure and position of methoxyl groups were crucial for the

anti-inflammatory effect, while the halogens and alkoxyl group show an inconclusive role in bioactivity.

2.4 Assessment of cytotoxicity and chemical stability of active compounds

To assess the safety of the synthetic C66 analogs, three active compounds **4a11**, **4a16**, and **4a17**, were tested for their hepatotoxicity in the human normal hepatic cell line (HL-7702) by MTT at a concentration of 10 μM. The survival rates of the cells with the administrated compounds at 24 h were determined (Fig. 2A). It was found that there was no significant difference between the compounds and the DMSO control group, indicating that they are relatively safe. Additionally, the chemical stability of these three compounds were further detected and measured by UV/Vis absorption in phosphate buffer (pH 7.4). As shown in Figure 2B, 50% of the curcumin degraded within 25 minutes at the maximum absorbance of 425 nm, this is consistent with its instability and poor metabolic property. However, **4a11**, **4a16**, and **4a17** demonstrated almost complete stability under the same condition (Fig. 2C-E). Overall, these result indicated that the modified MACs are chemically more stable than curcumin *in vitro*.

2.5 Active compounds inhibit TNF-α and IL-6 release in a dose-dependent manner

Among the tested compounds, **4a11**, **4a16**, and **4a17** were the most promising and were selected for further evaluation for their dose-dependent inhibitory effects against LPS-induced TNF-α and IL-6 release. RAW264.7 macrophages were pretreated with three active analogs in a series of concentrations (1, 2.5, 5.0, 10 and 20 μM) for 30 minutes and were then further incubated with LPS (0.5 μg/mL) for 24 h. The release of TNF-α and IL-6 in the culture medium was determined by ELISA. As shown in Figure 3, pretreatment with **4a11**, **4a16** and **4a17** exhibited a dose-dependent inhibition of both TNF-α and IL-6 release induced by LPS. Accordingly, the IC_{50} values of these compounds were calculated with all of them being under 2.0 μM except that of analogs **4a17**, and **4a11** which exhibited the lowest IC_{50} value (0.62 μM) when inhibiting TNF-α release. The dose-dependent inhibition as well as relatively low IC_{50} values by **4a11** and **4a16** suggests that they have potential in being a new anti-inflammatory agent.

2.6 Effect of 4a11 and 4a16 on LPS-induced lung permeability

The lung wet-to-dry (W/D) ratio and the total protein concentration in the BALF were evaluated at 6 h after LPS administration. Figure 4A and B show the lung W/D ratio and the total protein concentration in the BALF. As expected, the values of the LPS group were found to be higher than compared to those of the control group. Pretreatment with C66 and its active analogs **4a11** and **4a16** at 20 mg/kg, however, were found to effectively decrease lung W/D ratio and the total protein concentration.

To evaluate the histological changes after the new asymmetric C66 analogs were treated in LPS-treated rats, lung sections were subjected to histological assessment after 6 h from first injection of LPS with or without treatment. As shown in Figure 4C, Lung tissues from the control rats showed normal structures and no histopathological changes. In contrast, lung tissues from LPS administrated rats showed significant pathological changes, including lung edema, alveolar wall thickening and inflammatory cell infiltration. However, these histopathological changes were ameliorated in the C66 and its analogs (**4a11** and **4a16)** groups, particularly **4a11**, which demonstrated almost complete amelioration under the same condition. This result indicates that these modified MACs have a remarkable protective effect from lung-based injury in a rats model of ALI.

2.7 Effect of 4a11 and 4a16 analogs on cytokine production in BALF of LPS-induced ALI rats

To examine the anti-inflammatory actions of C66 and its analogs, the levels of proinflammatory cytokines and neutrophils were measured in BALF collected from animals. The data presented demonstrates that C66 could significantly inhibit the number of the neutrophils in BALF of LPS-induced ALI rats, whereas it's analogs, **4a11** and **4a16**, exhibited only weak to moderate inhibitions (Fig. 5A). As shown Figure 5B, administration of these symmetric or asymmetric MACs could effectively down-regulate the production of TNF-α compared to the LPS group. Therefore, these results indicated that synthetic C66 analogs, **4a11** and **4a16**, might play a direct protective role

in LPS-induced pulmonary inflammation by inhibition of inflammatory cytokines and neutrophils. To verify our results, we further enriched our immunohistochemical analysis with CD68, a macrophage marker. As shown in **Figure 5C**, LPS alone causes a significant accumulation of macrophage in the lung sections, whereas there was no significant difference in the number of CD68-stained macrophages between treated and untreated groups. Our results showed that administration of **4a11** and **4a16** resulted in a significant therapeutic effect on LPS-induced pulmonary inflammation.

2.8 Effects of 4a11 and 4a16 on mRNA expression of pro-inflammatory cytokines in Beas-2B cells

To further evaluate the anti-inflammatory potential of these C66 analogs, we investigated the effects of **4a11** and **4a16** in the proinflammatory cytokine mRNA expression of LPS-stimulated Beas-2B cells. Beas-2B cells were treated with LPS (1.0 μg/mL) for 6 h and examined for the expression of proinflammatory genes with or without the compounds present using RT-qPCR study. As shown in **Figure 6**, LPS significantly increased the level TNF-α, IL-6, IL-1β, COX-2, ICAM-1 and VCAM-1 mRNA expression compared to those of the vehicle control, while **4a11** and **4a16** potently inhibited LPS-induced up-regulation of all tested proinflammatory genes with a statistical significance. Importantly, **4a11** and **4a16**, were more potent in suppression of IL-1β, COX-2, ICAM-1 and VCAM-1 expressions than the lead compound C66. These data suggest that C66 analogs are potent inhibitors of LPS-induced mRNA overexpression of inflammatory genes in Beas-2B cells.

3. Conclusion

In summary, we designed and synthesized a series of symmetric mono-carbonyl analogs of curcumin based on C66 as the lead molecule. Among the new synthetic compounds, cyclopentanone-containing analogs were more effective preventing in LPS-induced IL-6 and TNF-α release in RAW 264.7 macrophages. Meanwhile, a SAR study revealed that the position of the methoxyl group in the aromatic ring was crucial for an anti-inflammatory effect, while the halogens

and alkoxyl group showed circumstantial roles in bioactivity. Subsequently, the promising compounds **4a11**, **4a16** and **4a17** were found to display excellent chemical stability and safety *in vitro*, as well as the dose-dependent inhibition against the release of TNF-α and IL-6. Also, **4a11** and **4a16** were shown to have a remarkable protective effect against pulmonary inflammation for the therapeutic action in LPS-induced acute lung injury. Furthermore, **4a11** and **4a16** potently inhibited the up-regulation of TNF-a, IL-6, IL-1b, COX-2, ICAM-1 and VCAM-1 in LPS-stimulated Beas-2B cells. Altogether, these results suggest that novel asymmetric C66 analogs might serve as potential agents for the treatment of LPS-induced acute lung injury.

4. Experimental section

4.1. General

Unless otherwise noted, all materials were obtained from commercial suppliers and were used without further purification. Melting points were determined on a Fisher-Johns melting apparatus without correction. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on Bruker 600 MHz instruments. The chemical shifts were presented in terms of parts per million with TMS as the internal reference. Electron-spray ionization mass spectra in positive mode (ESI-MS) data were recorded on a Bruker Esquire 3000t spectrometer. Analytical thin-layer chromatography was performed on Merck silica gel 60-F_{254} plates (Merck). Flash chromatography was accomplished on Merck silica gel (230-400 mesh).Visualization was accomplished with 254 nm UV light and phosphomolybdic acid (PMA) or potassium permanganate staining followed by heating. The identification of samples from different experiments was secured by mixed melting points and superimposable NMR spectra.

4.2 Chemistry

4.2.1 Preparation of the intermediates 3a and 3b

A solution of cyclopentanone **1a** or cyclohexanone **1b** (0.1 mol), morpholine (10.45 g, 0.12 mol) and 4-toluenesulfonic acid (0.04 g, 0.23 mmol) in dry toluene (30 mL) was stirred at 110°C for 6 h.

At the end of this time, the solvent was removed *in vacuo* to give morpholine enamine **2a** or **2b** without further purification. A solution of enamine **2a** or **2b** (0.027 mol) in EtOH (20 mL) and 2-(trifluoromethyl)benzaldehyde (4.0 g, 0.023 mol) was stirred under reflux for 6 h. After this period, the resulting mixture was acidized to a pH of 3-4 by a solution of 5% HCl, diluted with brine and extracted with EtOAc (3×10 mL). The combined organic layers were washed with brine and dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was further purified by flash column chromatography to give the intermediates **3a** and **3b**.

4.2.1.1 (*E*)-2-[(Trifluoromethyl)benzylidene] cyclopentanone (3a)

White powder, 79.7% yield, m.p: 81.9-83.2 °C. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm) 7.73 (1H, d, *J* = 7.8 Hz, H-3), 7.66 (1H, s, H-β), 7.56 (1H, t, *J* = 7.2 Hz, H-5), 7.54 (1H, d, *J* = 7.8 Hz, H-6), 7.44 (1H, t, *J* = 7.2 Hz, H-4), 2.83 (2H, t, *J* = 6.6 Hz, Cyclopentanone-$CH_2CH_2CH_2$-), 2.44 (2H, t, *J* = 7.8 Hz, Cyclopentanone-$CH_2CH_2CH_2$-), 1.98-2.00 (2H, m, Cyclopentanone-$CH_2CH_2CH_2$-).

4.2.1.2 (*E*)-2-[(Trifluoromethyl)benzylidene]cyclohexanone (3b)

Yellow oil, 51.7% yield. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm) 7.69 (1H, d, *J* = 7.8 Hz, H-3), 7.54 (1H, s, H-β), 7.52 (1H, d, *J* = 7.2 Hz, H-6), 7.41 (1H, t, *J* = 7.8 Hz, H-5), 7.26 (1H, t, *J* = 4.2 Hz, H-4), 2.54 (4H, t, *J* = 4.8 Hz, Cyclohexanone-$CH_2CH_2CH_2CH_2$-), 1.90-1.94 (2H, m, Cyclohexanone-$CH_2CH_2CH_2CH_2$-), 1.72-1.74 (2H, m, Cyclohexanone-$CH_2CH_2CH_2CH_2$-).

4.2.2 General procedure for preparation of 4a1-a8, a10-a15, a17, b1-b15, b18-b23

Aqueous sodium hydroxide solution (20% w/v, 1 mL) was added to a solution of **3a** or **3b** (0.40 mmol) and the appropriate arylaldehyde (0.40 mmol) in EtOH (5 mL). The reaction mixture was stirred at room temperature for 24 h. The resulting solution was then diluted with brine and extracted with EtOAc (3×10 mL). The combined organic layers were washed with brine and dried over

anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was further purified by flash column chromatography to furnish target products.

4.2.2.1 (2*E*,5*E*)-2-[4-(Allyloxy)benzylidene]-5-[2-(trifluoromethyl)benzylidene]cyclopentanone (4a1) :

Yellow powder, 31.9% yield, mp: 126.2-128.0 °C. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm) 7.92 (1H, s, H-β), 7.79 (1H, s, H-β'), 7.71 (1H, d, *J* = 7.8 Hz, H-3), 7.54 (1H, t, *J* = 7.2 Hz, H-5), 7.46 (2H, d, *J* = 9.0 Hz, H-2', H-6',), 7.42 (1H, t, *J* = 7.8 Hz, H-4), 7.32 (1H, d, *J* = 7.8 Hz, H-6), 6.95 (2H, d, *J* = 8.4 Hz, H-3', H-5'), 6.03-6.09 (1H, m, Ar-OCH_2CHCH_2), 5.43 (2H, d, *J* = 5.4 Hz, Ar-OCH_2CHCH_2), 5.32 (1H, d, *J* = 8.0 Hz, Ar-OCH_2CHCH-H), 4.58 (2H, d, *J* = 5.4 Hz, Ar-OCH_2CHCH-H)), 2.92 (2H, t, *J* = 6.0 Hz, Cyclopentanone-CH_2CH_2-), 2.62 (2H, t, *J* = 5.4 Hz, Cyclopentanone-CH_2CH_2-). ESI-MS m/z: 385.6 $(M+H)^+$.

4.2.2.2 (2*E*,5*E*)-2-[2-(Trifluoromethyl)benzylidene]-5-(2,4,6-trimethoxybenzylidene)cyclopentanone (4a2) :

Yellow powder, 41.9% yield, m.p: 83.7-85.3 °C. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm) 7.77 (1H, s, H-β), 7.72 (1H, d, *J* = 7.8 Hz, H-3), 7.70 (1H, t, *J* = 3.0 Hz, H-5), 7.65 (1H, s, H-β'), 7.55 (1H, d, *J* = 3.6 Hz, H-6), 7.42 (1H, t, *J* = 4.2 Hz, H-4), 6.15 (2H, s, H-3', H-5'), 3.86 (3H, s, 4'-OCH_3), 3.83 (6H, s, 2'-OCH_3, 6'-OCH_3), 2.81 (2H, t, *J* = 7.8 Hz, Cyclopentanone-CH_2CH_2-), 2.64 (2H, t, *J* = 7.8 Hz, Cyclopentanone-CH_2CH_2-). ESI-MS m/z: 419.5 $(M+H)^+$.

4.2.2.3 (2*E*,5*E*)-2-(2-Bromobenzylidene)-5-[2-(trifluoromethyl)benzylidene]cyclopentanone (4a3) :

Yellow powder, 44.3% yield, mp: 88.7-90.9 °C. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm) 7.867 (1H, s, H-β), 7.74 (2H, d, *J* = 7.8 Hz, H-3, H-3'), 7.67 (1H, d, *J* = 8.4 Hz, H-6), 7.58 (1H, s, H-β'),7.52 (1H, d,

J = 7.8 Hz, H-6'), 7.46 (2H, t, *J* = 7.8 Hz, H-5, H-5'), 7.36 (1H, t, *J* = 7.8 Hz, H-4), 7.22 (1H, t, *J* = 7.8 Hz, H-4'), 2.95 (4H, t, *J* = 3.0 Hz, Cyclopentanone-CH_2CH_2-). ESI-MS m/z: 409.5 $(M)^+$.

4.2.2.4 (2*E*,5*E*)-2-(2-Chlorobenzylidene)-5-[2-(trifluoromethyl)benzylidene]cyclopentanone (4a4) :

Yellow powder, 40.1% yield, mp: 104.8-107.7 °C. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm) 7.83 (1H, s, H-β), 7.74 (1H, d, *J* = 7.8 Hz, H-3), 7.59 (2H, t, *J* = 7.2 Hz, H-5, H-5'), 7.55 (1H, t, *J* = 2.4Hz, H-4'), 7.45 (1H, t, *J* = 7.2 Hz, H-4),7.22 (1H, d, *J* = 2.4 Hz, H-3'), 7.14 (1H, d, *J* = 8.4Hz, H-6'), 6.92 (1H, d, *J* = 8.4 Hz, H-6), 5.67 (1H, s, H-β'), 3.04 (2H, t, *J* = 3.0 Hz, Cyclopentanone-CH_2CH_2-), 2.98 (2H, t, *J* = 6.6 Hz, Cyclopentanone-CH_2CH_2-). ESI-MS m/z: 363.3 $(M+H)^+$.

4.2.2.5 (2*E*,5*E*)-2-(3,4-Dimethoxybenzylidene)-5-[2-(trifluoromethyl)benzylidene]cyclopentanone (4a5) :

Yellow powder, 50.3% yield, mp: 148.4-150.3 °C. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm) 7.84 (1H, s, H-β), 7.74 (1H, d, *J* = 7.8 Hz, H-3), 7.67 (1H, t, *J* = 7.8 Hz, H-5), 7.59 (1H, s, H-β'), 7.59 (1H, d, *J* = 2.4 Hz, H-6), 7.51 (1H, t, *J* = 7.8 Hz, H-4), 7.23 (1H, d, *J* = 6.6 Hz, H-6'), 7.12 (1H, s, H-2'), 6.94 (1H, d, *J* = 8.4 Hz, H-5'), 3.93 (6H, s, 3'-OCH_3, 4'-OCH_3), 3.07 (2H, t, *J* = 6.6 Hz, Cyclopentanone-CH_2CH_2-), 2.98 (2H, t, *J* = 6.6 Hz, Cyclopentanone-CH_2CH_2-). ESI-MS m/z: 389.2 $(M+H)^+$.

4.2.2.6 (2*E*,5*E*)-2-(Thiophen-2-ylmethylene)-5-[2-(trifluoromethyl)benzylidene]cyclopentanone (4a6) :

Yellow powder, 70.8% yield, mp: 147.6-149.1 °C. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm) 7.88 (1H, d, *J* = 3.0 Hz, H-5'), 7.87 (1H, s, H-β), 7.76 (1H, d, *J* = 7.8 Hz, H-3'), 7. 59 (1H, d, *J* = 6.6 Hz, H-3), 7.57 (1H, s, H-β'), 7.56 (1H, t, *J* = 4.8 Hz, H-4'), 7.46 (1H, t, *J* = 7.8 Hz, H-5), 7.42 (1H, d, *J* = 3.6 Hz,

H-6), 7.18 (1H, t, *J* = 4.2 Hz, H-4), 3.01 (2H, t, *J* = 6.0 Hz, Cyclopentanone-CH_2CH_2-), 2.97 (2H, t, *J* = 6.6 Hz, Cyclopentanone-CH_2CH_2-). ESI-MS m/z: 335.5 $(M+H)^+$.

4.2.2.7 (2*E*,5*E*)-2-[(1-Methyl-1H-pyrrol-2-yl)methylene]-5-[2-(trifluoromethyl)benzylidene]-cyclopentanone (4a7) :

Red powder, 64.5% yield, mp: 156.8-164.3 °C. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm) 7.79 (1H, s, H-β), 7.74 (1H, d, *J* = 7.8 Hz, H-3), 7.65 (1H, s, H-β'), 7. 63 (1H, d, *J* = 7.8 Hz, H-6), 7.58 (1H, t, *J* = 7.8 Hz, H-5), 7.44 (1H, t, *J* = 7.8 Hz, H-4), 6.88 (1H, d, *J* = 1.8 Hz, H-5'), 6.64 (1H, d, *J* = 3.0 Hz, H-3'), 6.30 (1H, t, *J* = 3.0 Hz, H-4'), 2.98 (2H, t, *J* = 7.2 Hz, Cyclopentanone-CH_2CH_2-), 3.80 (3H, s, 1'-CH_3), 2.88 (2H, t, *J* = 7.2 Hz, Cyclopentanone-CH_2CH_2-). ESI-MS m/z: 332.2 $(M+H)^+$.

4.2.2.8 (2*E*,5*E*)-2-[(*1H*-Indol-3-yl)methylene]-5-[2-(trifluoromethyl)benzylidene]cyclopean-tanone (4a8) :

Red oil, 32.5% yield. ^{1}H NMR (600 MHz, DMSO-*d6*): δ (ppm) 7.91 (1H, s, H-2'), 7.89 (1H, s, H-β), 7.85 (1H, d, *J* = 7.8 Hz, H-4'), 7.72 (1H, d, *J* = 7.8 Hz, H-3), 7.61 (1H, t, *J* = 7.2 Hz, H-5), 7.56 (1H, s, H-β'), 7.50 (1H, d, *J* = 8.4 Hz, H-6), 7.23 (1H, t, *J* = 7.2 Hz, H-4), 7.20 (1H, d, *J* = 7.2 Hz, H-7'), 7.16 (1H, t, *J* = 7.2 Hz, H-5'), 6.99 (1H, t, *J* = 6.6 Hz, H-6'), 3.07 (2H, t, *J* = 6.0 Hz, Cyclopentanone-CH_2CH_2-), 2.95 (2H, t, *J* = 6.0 Hz, Cyclopentanone-CH_2CH_2-). ESI-MS m/z: 368.3$(M+H)^+$.

4.2.2.9 (2*E*,5*E*)-2-[(*1H*-Indol-5-yl)methylene]-5-[2-(trifluoromethyl)benzylidene]cyclopenta-none (4a9) :

Red oil, 33.4% yield. ^{1}HNMR (600 MHz, DMSO-*d6*): δ (ppm) 8.34 (1H, s, -NH), 8.18 (1H, s, H-4'), 7.94 (1H, s, H-β), 7.84 (2H, d, *J* = 7.8 Hz, H-6', H-7'), 7.74 (1H, d, *J* = 7.8 Hz, H-3), 7.60 (1H, t, *J* =

7.8 Hz, H-5), 7.60 (1H, s, H-β'), 7.49 (1H, d, J = 8.4 Hz, H-6), 7.43 (1H, d, J = 7.2 Hz, H-2'), 7. 32 (1H, t, J = 2.4 Hz, H-4), 6.62 (1H, d, J = 8.4 Hz, H-3'), 3.14 (2H, t, J = 7.2 Hz, Cyclopentanone-CH_2CH_2-), 2.99 (2H, t, J = 7.2 Hz, Cyclopentanone-CH_2CH_2-). ESI-MS m/z: 368.4 $(M+H)^+$.

4.2.2.10 (2*E*,5*E*)-2-[(6-Bromobenzo[*d*][1,3]dioxol-5-yl)methylene]-5-[2-(trifluoromethyl)-benzylidene]cyclopentanone (4a10) :

Yellow powder, 41.9% yield, mp: 179.2-181.2 °C. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm) 7.89 (2H, s, H-β, H-β'), 7.75 (1H, d, J = 8.4 Hz, H-3), 7.58 (1H, t, J = 9.0 Hz, H-5), 7. 58 (1H, s, H-3'), 7.47 (1H, t, J = 7.2 Hz, H-4), 7.46 (1H, s, H-7'), 7.42 (1H, d, J = 7.8 Hz, H-6), 7.25 (2H, s, Ar-OCH_2-), 2.94 (4H, t, J = 4.2 Hz, Cyclopentanone-CH_2CH_2-). ESI-MS m/z: 451.0 $(M)^+$.

4.2.2.11 (2*E*,5*E*)-2-(2-Methoxybenzylidene)-5-[2-(trifluoromethyl)benzylidene]cyclopenta-none (4a12) :

Yellow powder, 61.9% yield, mp: 128.7-130.6 °C. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm) 8.04 (1H, s, H-β), 7.84 (1H, s, H-β'), 7.73 (1H, d, J = 7.8 Hz, H-3), 7.58 (1H, d, J = 3.6 Hz, H-6'), 7.57 (1H, t, J = 3.6 Hz, H-5), 7.51 (1H, d, J = 6.6 Hz, H-6), 7.45 (1H, t, J = 8.4 Hz, H-4), 7.36 (1H, t, J = 8.4 Hz, H-4'), 6.99 (1H, t, J = 7.8 Hz, H-5'), 6.94 (1H, d, J = 8.4 Hz, H-3'), 3.88 (3H, s, Ar-OCH_3), 2.99 (2H, t, J = 6.0 Hz, Cyclopentanone-CH_2CH_2-), 2.94 (2H, t, J = 3.6 Hz, Cyclopentanone-CH_2CH_2-). ESI-MS m/z: 359.3 $(M+H)^+$.

4.2.2.12 (2*E*,5*E*)-2-[2-(Trifluoromethyl)benzylidene]-5-[4-(trifluoromethyl)benzylidene]cyclo-pentanone (4a13) :

Yellow powder, 53.4% yield, mp: 130.1-132.5 °C. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm) 7.86 (1H, s, H-β), 7.77 (1H, d, *J* = 7.8 Hz, H-3), 7.66 (4H, d, *J* = 7.2 Hz, H-2', H-3', H-5', H-6'), 7.66 (1H, s, H-β'), 7.62 (1H, d, *J* = 5.4 Hz, H-6), 7.61 (1H, t, *J* = 5.4 Hz, H-5), 7.48 (1H, t, *J* = 4.2 Hz, H-4), 3.08 (2H, t, *J* = 4.8 Hz, Cyclopentanone-CH_2CH_2-), 3.01 (2H, t, *J* = 4.2 Hz, Cyclopentanone-CH_2CH_2-). ESI-MS m/z: 397.8 $(M+H)^+$.

4.2.2.13 (2*E*,5*E*)-2-(3-Bromo-4-fluorobenzylidene)-5-[2-(trifluoromethyl)benzylidene]cyclo-pentanone(4a14) :

Yellow powder, 45.9% yield, mp: 143.2-150.6 °C. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm) 7.87 (1H, s, H-β), 7.78 (1H, d, *J* = 6.0 Hz, H-6'), 7.75 (1H, d, *J* = 7.8 Hz, H-3), 7.59 (1H, d, *J* = 3.0 Hz, H-5'), 7.59 (1H, s, H-2'), 7.51 (1H, t, *J* = 2.4 Hz, H-5), 7.50 (1H, s, H-β'), 7.49 (1H, d, *J* = 2.4 Hz, H-6), 7.19 (1H, t, *J* = 8.4 Hz, H-4), 3.04 (2H, t, *J* = 6.6 Hz, Cyclopentanone-CH_2CH_2-), 2.98 (2H, t, *J* = 6.6 Hz, Cyclopentanone-CH_2CH_2-). ESI-MS m/z: 427.0 $(M)^+$.

4.2.2.14 (2*E*,5*E*)-2-(4-Chloro-3-nitrobenzylidene)-5-[2-(trifluoromethyl)benzylidene]cyclo-pentanone (4a15) :

Yellow oil, 35.6% yield. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm) 7.67 (1H, d, *J* = 7.8 Hz, H-6'), 7.59 (1H, s, H-2'), 7.52 (1H, t, *J* = 7.2 Hz, H-5), 7.42 (1H, t, *J* = 7.8 Hz, H-4), 7.33 (1H, d, *J* = 3.0 Hz, H-5'), 7.31 (1H, s, H-β), 7.13 (1H, s, H-β'), 6.88 (2H, d, *J* = 5.4 Hz, H-3, H-6), 3.11 (2H, t, *J* = 5.4 Hz, Cyclopentanone-CH_2CH_2-), 2.95 (2H, t, *J* = 6 Hz, Cyclopentanone-CH_2CH_2-).

4.2.2.15 (2*E*,5*E*)-2-{4-[3-(Dimethylamino)propoxy]benzylidene}-5-[2-(trifluoromethyl)benzy-lidene]cyclopentanone (4a16) :

Yellow oil, 40.8% yield. ^{1}HNMR (600 MHz, DMSO-*d6*): δ (ppm) 7.73 (1H, s, H-β), 7.68 (2H, d, *J* = 8.4 Hz, H-2', H-6'), 7.53 (1H, d, *J* = 9.0 Hz, H-3), 7.46 (1H, t, *J* = 7.2 Hz, H-5), 7.45 (1H, d, *J* = 8.4 Hz, H-6), 7.27 (1H, t, *J* = 7.8 Hz, H-4), 7.14 (1H, d, *J* =9.0 Hz, H-6), 6.98 (2H, d, *J* = 9.0 Hz, H-3', H-5'), 4.04 [2H, t, *J* = 6.0 Hz, Ar-$OCH_2CH_2CH_2N(CH_3)_2$], 2.85-2.89 [2H, m, Ar-$OCH_2CH_2CH_2N(CH_3)_2$], 2.74 [2H, t, *J* = 6.0 Hz, Ar-$OCH_2CH_2CH_2N(CH_3)_2$], 2.68 (2H, t, *J* = 6.0 Hz, Cyclopentanone-CH_2CH_2-), 2.63 (2H, t, *J* = 6.0 Hz, Cyclopentanone-CH_2CH_2-), 2.13 [6H, s, Ar-$OCH_2CH_2CH_2N(CH_3)_2$]. ESI-MS m/z: 430.2 $(M+H)^+$.

4.2.2.16 (2*E*,5*E*)-2-(2,3-Dimethoxybenzylidene)-5-[2-(trifluoromethyl)benzylidene]cyclo-pentanone (4a17) :

Yellow powder, 43.8% yield, mp: 60.3-62.5 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) 7.98 (1H, s, H-β), 7.87 (1H, s, H-β'), 7.76 (1H, d, *J* = 7.8 Hz, H-3), 7.60 (1H, d, *J* = 2.4 Hz, H-6), 7.60 (1H, t, *J* = 2.4 Hz, H-5), 7.47 (1H, t, *J* = 8.4 Hz, H-4), 7.156 (1H, d, *J* = 7.8 Hz, H-6'), 7.12 (1H, t, *J* = 8.4 Hz, H-5'), 6.98 (1H, d, *J* = 7.2 Hz, H-4'), 3.89 (6H, s, 2'-OCH_3, 3'-OCH_3), 3.00 (2H, t, *J* = 3.6 Hz, Cyclopentanone-CH_2CH_2-), 2.94 (2H, t, *J* = 3.6 Hz, Cyclopentanone-CH_2CH_2-). ESI-MS m/z: 389.1 $(M+H)^+$.

4.2.2.17 (2*E*,6*E*)-2-[4-(Allyloxy)benzylidene]-6-[2-(trifluoromethyl)benzylidene]cyclo-hexanone (4b1):

Yellow powder, 70.2% yield, mp: 82.3-84.7 °C. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm) 7.92 (1H, s, H-β), 7.79 (1H, s, H-β'), 7.71 (1H, d, *J* = 7.8 Hz, H-3), 7.54 (1H, t, *J* = 7.8 Hz, H-5), 7.45 (2H, d, *J* = 8.4 Hz, H-2', H-6'), 7.42 (1H, t, *J* = 7.8 Hz, H-4), 7.32 (1H, d, *J* = 7.8 Hz, H-6), 6.95 (2H, d, *J* = 9.0 Hz, H-3', H-5'), 6.01-6.09 (1H, m, Ar-OCH_2CHCH_2), 5.43 (1H, d, *J* = 10.2 Hz, Ar-OCH_2CHCH_2), 5.32 (1H, d, *J* = 10.2 Hz, Ar-OCH_2CHCH_2), 4.58 (2H, d, *J* = 5.4 Hz, Ar-OCH_2CHCH_2), 2.92 (2H, t, *J* = 6.0 Hz, Cyclohexanone-$CH_2CH_2CH_2$-), 2.62 (2H, t, *J* = 5.4 Hz, Cyclohexanone-$CH_2CH_2CH_2$-), 1.75-1.77 (2H, m, Cyclohexanone-$CH_2CH_2CH_2$-). ESI-MS m/z: 399.3 $(M+H)^+$.

4.2.2.18 (2*E*,6*E*)-2-[2-(Trifluoromethyl)benzylidene]-6-(2,4,6-trimethoxybenzylidene)cyclo-hexanone (4b2):

Yellow powder, 40.5% yield, mp: 88.5-90.4 °C. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm) 7.87 (1H, s, H-β), 7.84 (1H, s, H-β'), 7.62 (1H, d, *J* = 8.4 Hz, H-3), 7.53 (1H, t, *J* = 7.8 Hz, H-5), 7.40 (1H, t, *J* = 6.6 Hz, H-4), 7.31 (1H, d, *J* = 7.2 Hz, H-6), 6.15 (1H, s, H-3'), 6.12 (1H, s, H-5'), 3.81 [9H, s, Ar-$(OCH_3)_3$], 2.60 (2H, t, *J* = 7.2 Hz, Cyclohexanone-$CH_2CH_2CH_2$-), 2.46 (2H, t, *J* = 6.6 Hz, Cyclohexanone-$CH_2CH_2CH_2$-), 1.65-1.67 (2H, m, Cyclohexanone-$CH_2CH_2CH_2$-). ESI-MS m/z: 433.8 $(M+H)^+$.

4.2.2.19 (2*E*,6*E*)-2-(2-Bromobenzylidene)-6-[2-(trifluoromethyl)benzylidene]cyclohexanone (4b3):

Yellow powder, 71.9% yield, mp: 86.3-88.5 °C. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm) 7.96 (1H, s, H-β), 7.85 (1H, s, H-β'), 7.72 (1H, d, *J* = 12.0 Hz, H-3), 7.64 (1H, d, *J* = 7.8 Hz, H-3'), 7.55 (1H, t, *J* =7.2 Hz, H-5), 7.31 (1H, t, *J* = 7.8 Hz, H-5'), 7.32 (1H, t, *J* = 1.8 Hz, H-4), 7.31 (2H, d, *J* = 5.4 Hz, H-6, H-6'), 7.19 (1H, t, *J* = 6.0 Hz, H-4'), 2.74 (2H, t, *J* = 6.6 Hz, Cyclohexanone-$CH_2CH_2CH_2$-), 2.64 (2H, t, *J* = 5.4 Hz, Cyclohexanone-$CH_2CH_2CH_2$-), 1.71-1.75 (2H, m, Cyclohexanone-$CH_2CH_2CH_2$-). ESI-MS m/z: 421.1 $(M)^+$.

4.2.2.20 (2*E*,6*E*)-2-(2-Chlorobenzylidene)-6-[2-(trifluoromethyl)benzylidene]cyclohexanone (4b4):

Yellow powder, 61.7% yield, mp: 83.0-85.5 °C. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm) 7.96 (1H, s, H-β), 7.92 (1H, s, H-β'), 7.72 (1H, d, *J* = 12.0 Hz, H-3), 7.64 (1H, d, *J* = 7.8 Hz, H-3'), 7.45 (1H, t, *J* = 5.6 Hz, H-5), 7.33 (1H, t, *J* = 4.2 Hz, H-5'), 7.28 (1H, t, *J* = 3.0 Hz, H-4), 7.28 (2H, d, *J* = 2.4 Hz, H-6,

H-6'), 7.27 (1H, t, *J* = 6.0 Hz, H-4'), 2.76 (2H, t, *J* = 5.4 Hz, Cyclohexanone-$CH_2CH_2CH_2$-), 2.64 (2H, t, *J* = 5.4 Hz, Cyclohexanone-$CH_2CH_2CH_2$-), 1.72-1.75 (2H, m, Cyclohexanone-$CH_2CH_2CH_2$-). ESI-MS m/z: 377.4 $(M+H)^+$.

4.2.2.21 (2*E*,6*E*)-2-(3,4-Dimethoxybenzylidene)-6-[2-(trifluoromethyl)benzylidene]cyclo-hexanone (4b5):

Yellow oil, 50.9% yield. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm) 7.96 (1H, s, H-β), 7.85 (1H, s, H-β'), 7.74 (1H, d, *J* = 7.8 Hz, H-3), 7.59 (1H, t, *J* = 3.0 Hz, H-5), 7.58 (1H, d, *J* = 3.0 Hz, H-6), 7.58 (1H, s, H-2'), 7.11 (1H, t, *J* = 7.8 Hz, H-4), 6.98 (1H, d, *J* = 1.2 Hz, H-6'), 6.97 (1H, d, *J* = 6.6 Hz, H-5'), 3.88 (3H, s, 3'-OCH_3), 3.87 (3H, s, 4'-OCH_3), 3.01 (2H, t, *J* = 2.4 Hz, Cyclohexanone-$CH_2CH_2CH_2$-), 2.94 (2H, t, *J* = 4.2 Hz, Cyclohexanone-$CH_2CH_2CH_2$-), 1.71-1.75 (2H, m, Cyclohexanone-$CH_2CH_2CH_2$-). ESI-MS m/z: 403.2 $(M+H)^+$.

4.2.2.22 (2*E*,6*E*)-2-(Thiophen-2-ylmethylene)-6-[2-(trifluoromethyl)benzylidene]cyclo-hexanone (4b6):

Yellow oil, 54.6% yield. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm) 7.99 (1H, s, H-β), 7.95 (1H, s, H-β'), 7.72 (1H, d, *J* = 7.8 Hz, H-5'), 7.56 (1H, d, *J* = 4.8 Hz, H-3'), 7.55 (1H, t, *J* = 6.0 Hz, H-4'), 7.43 (1H, t, *J* = 10.8 Hz, H-5), 7.38 (1H, d, *J* = 4.8 Hz, H-3), 7.31 (1H, t, *J* = 2.4 Hz, H-4), 7.14-7.17 (1H, m, H-6), 2.94 (2H, t, *J* = 6.0 Hz, Cyclohexanone-$CH_2CH_2CH_2$-), 2.62 (2H, t, *J* = 4.2 Hz, Cyclohexanone-$CH_2CH_2CH_2$-), 1.97-1.98 (2H, m, Cyclohexanone-$CH_2CH_2CH_2$-). ESI-MS m/z: 349.5 $(M+H)^+$.

4.2.2.23 (2*E*,6*E*)-2-[(1-Methyl-*1H*-pyrrol-2-yl)methylene]-6-[2-(trifluoromethyl)benzylidene]cyclohexanone (4b7):

Red oil, 20.9% yield. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm) 7.93 (1H, s, H-β), 7.87 (1H, s, H-β'), 7.71 (1H, d, *J* = 7.8 Hz, H-3), 7.54 (1H, t, *J* = 7.8 Hz, H-5), 7.42 (1H, t, *J* = 7.8 Hz, H-4), 7.31 (1H, t, *J* = 7.8 Hz, H-6), 6.85 (1H, d, *J* = 4.2 Hz, H-3'), 6.60 (1H, d, *J* = 3.6 Hz, H-5'), 6.23 (1H, t, *J* = 3.0 Hz, H-4'), 3.78 (3H, s, 1'-CH_3), 2.82 (2H, t, *J* = 5.4 Hz, Cyclohexanone-$CH_2CH_2CH_2$-), 2.61 (2H, t, *J* = 6.0 Hz, CycloHexanone-$CH_2CH_2CH_2$-), 1.77-1.82 (2H, m, Cyclohexanone-$CH_2CH_2CH_2$-). ESI-MS m/z: 346.1 $(M+H)^+$.

4.2.2.24 (2*E*,6*E*)-2-[(*1H*-Indol-3-yl)methylene]-6-[2-(trifluoromethyl)benzylidene]cyclohexan (4b8):

Yellow powder, 15.6% yield, m.p: 99.7-102.6 °C. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm): 7.95 (1H, s, H-1'), 7.92 (1H, s, H-β), 7.79 (1H, s, H-2'), 7.756 (1H,s, H-β') , 7.72 (1H, t, *J* = 7.2 Hz, H-5), 7.55 (1H, t, *J* = 7.2 Hz, H-7'), 7.44 (3H, d, *J* = 9.0 Hz, H-3, H-6, H-4'), 7.43 (1H, t, *J* = 7.8 Hz, H-4), 7.32 (2H, t, *J* = 7.2 Hz, H-5', H-6'), 2.62 (4H, t, *J* = 6.0 Hz, Cyclohexanone-$CH_2CH_2CH_2$-), 1.69-1.71 (2H, m, Cyclohexanone-$CH_2CH_2CH_2$-).

4.2.2.25 (2*E*,6*E*)-2-[(*1H*-Indol-5-yl)-methylene]-6-[2-(trifluoromethyl)benzylidene]cyclohexanone (4b9):

Red oil, 24.6% yield. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm) 10.04 (1H, s, H-1'), 8.18 (1H, s, H-4'), 8.04 (1H, s, H-β), 8.00 (1H, s, H-β'), 7.78 (3H, d, *J* = 8.4 Hz, H-3, H-6', H-7'), 7.48 (1H, d, *J* = 8.4 Hz, H-6), 7.32 (2H, t, *J* = 9.0 Hz, H-4, H-5), 6.78 (2H, d, *J* = 1.8 Hz, H-2', H-3'), 2.62 (4H, t, *J* = 6.0 Hz, Cyclohexanone-$CH_2CH_2CH_2$-), 1.69-1.71 (2H, m, Cyclohexanone-$CH_2CH_2CH_2$-). ESI-MS m/z: 383.2 $(M)^+$.

4.2.2.26 (2*E*,6*E*)-2-[(6-Bromobenzo[*d*][1,3]dioxol-5-yl)methylene]-6-[2-(trifluoromethyl)-benzylidene]cyclohexanone (4b10):

Yellow powder, 50.4% yield, mp: 182.5-184.6 °C. ^{1}HNMR (600 MHz, $CDCl_3$): δ(ppm) 7.923 (1H, s, H-β), 7.79 (1H, s, H-β'), 7.71 (1H, d, *J* = 1.8 Hz, H-3), 7.54 (1H, t, *J* = 7.2 Hz, H-5), 7.42 (1H, t, *J* = 7.8 Hz, H-4), 7.31 (1H, d, *J* = 7.2 Hz, H-6), 7.09 (1H, s, H-3'), 6.82 (1H, s, H-6'), 2.74 (4H, t, *J* = 6.0 Hz, Cyclohexanone-$CH_2CH_2CH_2$-), 1.72-1.74 (2H, m, Cyclohexanone-$CH_2CH_2CH_2$-). ESI-MS m/z: 466.8 $(M+H)^+$.

4.2.2.27 (2*E*,6*E*)-2-(4-Ethoxybenzylidene)-6-[2-(trifluoromethyl)benzylidene]cyclohexanone (4b12):

Yellow powder, 65.2% yield, mp: 98.4-100.2 °C. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm) 7.93 (1H, d, *J* = 7.8 Hz, H-3), 7.79 (1H, s, H-β), 7.72 (1H, t, *J* = 7.2 Hz, H-5), 7.53-7.56 (1H, m, H-2'), 7.46 (2H, d, *J* = 7.2 Hz, H-6, H-6'), 7.43 (1H, s, H-β'), 7.32 (1H, t, *J* = 7.8 Hz, H-4), 6.93 (1H, d, *J* = 4.2 Hz, H-3'), 6.92 (1H, d, *J* = 3.6 Hz, H-5'), 4.08 (2H, q, *J* =6.4 Hz,Ar-OCH_2CH_3), 2.92 (2H, t, *J* = 6.0 Hz, Cyclohexanone-$CH_2CH_2CH_2$-), 2.62 (2H, t, *J* = 6.0 Hz, Cyclohexanone-$CH_2CH_2CH_2$-), 1.71-1.81 (2H, m, Cyclohexanone-$CH_2CH_2CH_2$-), 1.44 (3H, t, *J* = 6.6 Hz, Ar-OCH_2CH_3), ESI-MS m/z: 387.7 $(M+H)^+$.

4.2.2.28 (2*E*,6*E*)-2-(2,5-Dimethylbenzylidene)-6-[2-(trifluoromethyl)benzylidene]cyclo-hexanone (4b13):

Yellow oil, 30.6% yield. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm) 7.94 (1H, s, H-β), 7.89 (1H, s, H-β'), 7.72 (1H, d, *J* = 7.8 Hz, H-3), 7.55 (1H, t, *J* = 7.8 Hz, H-5), 7.43 (1H, t, *J* = 7.8 Hz, H-4), 7.34 (1H, d, *J* = 7.8 Hz, H-6), 7.12 (1H, d, *J* = 8.4 Hz, H-3'), 7.06 (1H, s, H-6'), 7.05 (1H, d, *J* = 5.4 Hz, H-4'), 2.76 (2H, t, *J* = 7.8 Hz, Cyclohexanone-$CH_2CH_2CH_2$-), 2.63 (2H, t, *J* = 5.4 Hz,

Cyclohexanone-$CH_2CH_2CH_2$-), 2.33 (3H, s, 2'-CH_3), 2.29 (3H, s, 4'-CH_3), 1.69-1.72 (2H, m, Cyclohexanone-$CH_2CH_2CH_2$-). ESI-MS m/z: 371.3 $(M+H)^+$.

4.2.2.29 (2*E*,6*E*)-2-[4-(*tert*-Butyl)benzylidene]-6-[2-(trifluoromethyl)benzylidene]cyclo-hexanone (4b14):

Yellow powder, 31.6% yield, mp: 127.9-129.8 °C. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm) 7.93 (1H, s, H-β), 7.82 (1H, s, H-β'), 7.71 (1H, d, J = 7.8 Hz, H-3), 7.55 (1H, t, J = 7.2 Hz, H-5), 7.43 (4H, d, J = 3.0 Hz, H-2', H-3', H-5', H-6'), 7.43 (1H, t, J = 3.0 Hz, H-4), 7.32 (1H, d, J = 7.8 Hz, H-6), 2.94 (2H, t, J = 5.4 Hz, Cyclohexanone-$CH_2CH_2CH_2$-), 2.63 (2H, t, J = 5.4 Hz, Cyclohexanone-$CH_2CH_2CH_2$-), 1.74-1.76 (2H, m, Cyclohexanone-$CH_2CH_2CH_2$-), 1.31 [9H, s, 4'-$C(CH_3)_3$]. ESI-MS m/z: 399.2 $(M+H)^+$.

4.2.2.30 (2*E*,6*E*)-2-(3-Bromo-4-methoxybenzylidene)-6-[2-(trifluoromethyl)benzylidene]-cyclohexanone (4b15):

Yellow powder, 72.3% yield, mp: 113.2-115.4 °C. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm) 7.93 (1H, s, H-2'), 7.72 (1H, s, H-β), 7.71 (2H, d, J = 8.4 Hz, H-3, H-6'), 7.55 (1H, t, J = 4.8 Hz, H-5), 7.42 (1H, d, J = 5.4 Hz, H-6), 7.32 (1H, t, J = 7.2 Hz, H-4), 6.94 (1H, t, J = 8.4 Hz, H-5'), 3.94 (3H, s, 4'-OCH_3), 2.91 (2H, t, J = 6.6 Hz, Cyclohexanone-$CH_2CH_2CH_2$-), 2.62 (2H, t, J = 5.4 Hz, Cyclohexanone-$CH_2CH_2CH_2$-), 1.76-1.78 (2H, m, Cyclohexanone-$CH_2CH_2CH_2$-). ESI-MS m/z: 451.2 $(M)^+$.

4.2.2.31 (2*E*,6*E*)-2-(2-Fluoro-4-methoxybenzylidene)-6-[2-(trifluoromethyl)benzylidene]-cyclohexanone (4b17):

Yellow oil, 30.5% yield. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm) 7.94 (1H, s, H-β), 7.79 (1H, s, H-β'), 7.72 (1H, d, *J* = 7.8 Hz, H-3), 7.65 (1H, d, *J* = 8.4 Hz, H-6), 7.55 (1H, t, *J* = 7.2 Hz, H-5), 7.43 (1H, t, *J* = 7.8 Hz, H-4), 7.43 (1H, d, *J* = 7.8 Hz, H-6'), 7.79 (1H, s, H-β'), 7.33 (1H, s, H-3'), 7.32 (1H, d, *J* = 7.8 Hz, H-5'), 3.80 (3H, s, Ar-OCH_3), 2.80 (4H, t, *J* = 5.4 Hz, Cyclohexanone-$CH_2CH_2CH_2$-), 1.73-1.75 (2H, m, Cyclohexanone-$CH_2CH_2CH_2$). ESI-MS m/z: 391.4 $(M+H)^+$.

4.2.3 General procedure for synthesis of 4a11, 4b11 and 4b16

To a solution of enamine **2a** or **2b** (0.40 mmol) and corresponding arylaldehyde (0.40 mmol) in EtOH (5 mL) was catalyzed by dry hydrogen chloride gas at room temperature. After 24 h, the resulting solution was diluted with saturated aqueous $NaHCO_3$, the EtOH was then removed under reduced pressure and extracted with EtOAc (3×10 mL). The combined organic layers were washed with brine, dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was further purified by flash column chromatography to provide C66 analogs **4a11, 4b11** and **4b16**.

4.2.3.1 (2*E*,5*E*)-2-(4-Hydroxy-3-methoxybenzylidene)-5-[2-(trifluoromethyl)benzylidene]-cyclopentanone (4a11) :

Yellow powder, 55.9% yield, m.p: 133.1-134.8 °C. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm) 7.83 (1H, s, H-β), 7.74 (1H, d, *J* = 7.8 Hz, H-3), 7.59 (1H, s, H-β'), 7.58 (1H, t, *J* = 7.2 Hz, H-5), 7.57 (1H, d, *J* = 6.6 Hz, H-6), 7.45 (1H, t, *J* = 7.8 Hz, H-4), 7.19 (1H, d, *J* = 8.4 Hz, H-6'), 7.09 (1H, s, H-2'), 6.98 (1H, d, *J* = 8.4 Hz, H-5'), 5.91 (1H, s, 4'-OH), 3.95 (3H, s, 3'-OCH_3), 3.04 (2H, t, *J* = 4.8 Hz, Cyclopentanone-CH_2CH_2-), 2.98 (2H, t, *J* = 4.2 Hz, Cyclopentanone-CH_2CH_2-). ESI-MS m/z: 375.4 $(M+H)^+$.

4.2.3.2 (2*E*,6*E*)-2-(4-Hydroxy-3-methoxybenzylidene)-6-[2-(trifluoromethyl)benzylidene]-cyclo-hexanone (4b11):

Yellow oil, 31.6% yield. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm) 7.92 (1H, s, H-β), 7.77 (1H, s, H-β'), 7.71 (1H, d, *J* = 7.8 Hz, H-3), 7.54 (1H, t, *J* = 7.8 Hz, H-5), 7.43 (1H, d, *J* = 7.8 Hz, H-6'), 7.42 (1H, t, *J* =1.8 Hz, H-4), 7.42 (1H, s, H-2'), 7.31 (1H, d, *J* = 1.8 Hz, H-6), 7.09 (1H, d, *J* = 8.4 Hz, H-5'), 5.84 (1H, s, -OH), 3.93 (3H, s, $-OCH_3$), 2.93 (2H, t, *J* = 4.8 Hz, Cyclohexanone-$CH_2CH_2CH_2$-), 2.62 (2H, t, *J* = 5.4 Hz, Cyclohexanone-$CH_2CH_2CH_2$-), 1.75-1.78 (2H, m, Cyclohexanone-$CH_2CH_2CH_2$-). ESI-MS m/z: 389.3 $(M+H)^+$.

4.2.3.3 (2*E*,6*E*)-2-(3-Bromo-4-hydroxybenzylidene)-6-[2-(trifluoromethyl)benzylidene]-cyclohexanone (4b16) :

Yellow powder, 50.6% yield, mp: 83.2-84.4 °C. ^{1}HNMR (600 MHz, $CDCl_3$): δ (ppm) 9.83 (1H, s, -OH), 8.04 (1H, s, H-2'), 8.04 (1H, s, H-β'), 7.86 (1H, d, *J* = 10.8 Hz, H-3), 7.69 (1H, s, H-β'), 7.71 (1H, t, *J* = 7.8 Hz, H-5), 7.62 (1H, t, *J* = 1.8 Hz, H-4), 7.43 (1H, d, *J* = 5.4 Hz, H-6), 7.31 (1H, d, *J* = 7.8 Hz, H-6'), 7.06 (1H, d, *J* = 8.4Hz, H-5'), 2.89 (2H, t, *J* = 5.4 Hz, Cyclohexanone-$CH_2CH_2CH_2$-), 2.63 (2H, t, *J* = 5.4 Hz, Cyclohexanone-$CH_2CH_2CH_2$-), 1.71-1.75 (2H, m, Cyclohexanone-$CH_2CH_2CH_2$-). ESI-MS m/z 437.0 $(M)^+$.

4.3 Determination of TNF-α and IL-6.

After treatment of cells with indicated compounds and LPS, The levels of TNF-α and IL-6 in the media were determined with enzyme-linked immunosorbent assay (eBioScience, San Diego, CA) according to the manufacturer's instructions. The total amount of the inflammatory factor in the medium was normalized to the total protein quantity of the viable cell pellets

4.4 UV-visible absorption spectra of curcumin and synthetic MACs

Absorbance readings were taken from 250 to 600 nm using a spectrum Max M5 (Molecular Devices, USA). A stock solution of 1 mM curcumin or C66 analogs was prepared and diluted by

phosphate buffer (pH 7.4) to a final concentration of 20 μM. In the experiments where degradation of curcumin was recorded, the absorption spectra were collected for 25 min at 5 min intervals. The UV-visible absorbance spectrum was measured at 25 ^{o}C at varying time interval in a 1 cm path-length quartz cuvette.

4.5 Animals and preparation of acute lung injury (ALI) model

Male special pathogen free SD rats weighing 180–220 g were purchased from Animal Experimental Center, Wenzhou Medical University, China. Animals were housed in groups of five per standardcage,on 12 h light/dark cycle; and air temperature was maintained at 25 ^{o}C. Protocols involving the use of animals were approved by the Wenzhou Medical University Animal Policy and Welfare Committee (wydw2014-0062).

The rats were divided into five groups: (1) Normal control group, rats were received 50 μL of 0.9% saline by intratracheally injections; (2) Model control group, rats were intratracheally administered LPS (5 mg/kg). (3) Positive control group, C66 (20 mg/kg) was orally administrated 7 days before LPS challenge. (4) **4a11** treated group, **4a11** (20 mg/kg) was orally administrated 7 days before LPS challenge. (5) **4a16** treated group, **4a16** (20 mg/kg) was orally administrated 7 days before LPS challenge. To induced acute lung injury model, rats were intratracheally administered with 5 mg/kg LPS. Six hours after LPS challenge, rats were all anaesthetized by intraperitoneal injection of chloral hydrate.

4.6 Wet/Dry weight ratio

Rats were sacrificed at 6 h after LPS challenge. The upper lobe of right lung was excised, blotted dry, and weighed to obtain the "wet" weight and then placed in an oven at 65 ^{o}C for 48 h to obtain the "dry" weight. The ratio of the wet lung to the dry lung was calculated to assess tissue edema.

4.7 Protein concentration in bronchoalveolar lavage fluid (BALF)

BALF was obtained at the end of the experimental by irrigating the left lung with saline (3 × 1.5 mL). This fluid was centrifuged at 1000 rpm for 10 min, and the protein concentration in the supernatant was determined using a BCA protein assay (Pierce, Rockford, IL, USA).

4.8 Histological analysis of lung

Lung tissues were fixed in 4% paraformaldehyde (PFA), embedded in paraffin, and cut into 5 μmthick sections. Lung histologic specimens stained with hematoxylin and eosin (Richard Allen Scientific, Kalamazoo, MI) were assessed by a pathologist blinded to the study groups and scored by a system developed to grade lung injury. For CD68 immunostaining, tissue sections were de-paraffinized and incubated for 1h at room temperature with anti-CD68 antibody (Santa Cruz Biotechnology) to stain for macrophages. Briefly, lung injury was graded from 0 (normal) to 4 (severe) based on the following categories: neutrophil infiltration, interstitial edema, hemorrhage, and hyalinemembrane. The sum of all scores was combined to calculate a composite score as described previously.

4.9 RT-qPCR

Cells were homogenized in TRIZOL kit (Invitrogen, Carlsbad, CA) for extraction of RNA according to each manufacturer's protocol. Both reverse transcription and quantitative PCR were carried out using a two-step M-MLV Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen, Carlsbad, CA). Eppendorf Mastercycler ep realplex detection system (Eppendorf, Hamburg, Germany) was used for q-PCR analysis. The primers of genes were synthesized by Invitrogen. PCR primers were designed using Primer Premier Version 5.0 software (Premier Biosoft, Palo Alto, CA, USA) and sequences were as follows (Invitrogen):

Rat TNF-α sense: 5'- TACTCCCAGGTTCTCTTCAAGG -3';

Rat TNF-α antisense: 5'- GGAGGCTGACTTTCTCCTGGTA -3';

Rat IL-6 sense: 5'- GAGTTGTGCAATGGCAATTC -3';

Rat IL-6 antisense: 5'- ACTCCAGAAGACCAGAGCAG -3';

Rat IL-1β sense: 5'- CACCTCTCAAGCAGAGCACAG -3';

Rat IL-1β antisense: 5'- GGGTTCCATGGTGAAGTCAAC -3';

Rat COX-2 sense: 5'- CGGAGGAGAAGTGGGGTTTAGGAT -3';

Rat COX-2 antisense: 5'- TGGGAGGCACTTGCGTTGATGG -3';

Rat ICAM-1 sense: 5'- AGATCATACGGGTTTGGGCTTC -3';

Rat ICAM-1 antisense: 5'- TATGACTCGTGAAAGAAATCAGCTC -3';

Rat VCAM-1 sense: 5'- TTTGCAAGAAAAGCCAACATGAAAG -3';

Rat VCAM-1 antisense: 5'- TCTCCAACAGTTCAGACGTTAGC -3';

Rat β-actin sense: 5'- AAGTCCCTCACCCTCCCAAAAG -3';

Rat β-actin antisense: 5'- AAGCAATGCTGTCACCTTCCC -3'.

The amount of each gene was determined and normalized by the amount of β-actin.

4.10 Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). Student's t-test was employed to analyze the differences between sets of data. Statistics were performed using GraphPad Pro (GraphPad, San Diego, CA). P values less than 0.05 ($p < 0.05$) were considered indicative of significance. All experiments were repeated at least three times.

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Figure Legends

Table 1. Chemical structures of all synthetic C66 analogs.

Scheme 1. Structural design and synthetic route of asymmetric C66 analogs **4a1-17** and **4b1-17**. Reagents and conditions: **(a)** 4-Methylbenzenesulfonic acid, toluene, 110^{o}C, reflux, 4h; **(b)** EtOH, 78^{o}C, reflux, 5h, saturate HCl, 20-30%; **(c)** Various aromatic aldehydes, HCl gas (or 20% NaOH), EtOH, rt, 10h, 20-80%.

Figure 1. C66 and 34 synthetic analogs inhibited LPS-induced IL-6 **(A)** and TNF-α **(B)** secretion in RAW 264.7 macrophages. Macrophages were plated at a density of 4.0×10^5/plate at 37^{o}C and 5% CO_2 overnight. Cells were pre-treated with C66 or its analogs (10 μM) for 30min, then treated with LPS (0.5 μg/ml) for 24 h. TNF-α and IL-6 levels in the culture media were measured by ELISA and were normalized by the total protein. The results were expressed as the percent of LPS control. Each bar represents mean ± SE of 3-5 independent experiments. Statistical significance relative to LPS group was indicated, *$p < 0.05$, **$p < 0.01$.

Figure 2. The cytotoxic evaluation of active C66 analogs and their ultraviolet-visible absorption spectra in phosphate buffer (pH 7.4) containing 5% dimethyl sulfoxide.

Figure 3. Three active C66 analogs dose-dependently inhibited LPS-induced TNF-α and IL -6 secretion in RAW 264.7 macrophages. Macrophages were plated at a density of 4.0×10^5/plate overnight in 37°C and 5% CO_2. Cells were pretreated with active compounds in a series concentration of 1μM, 2.5 μM, 5 μM, 10 μM and 20 μM for 30 minutes and subsequently incubated with or without LPS (0.5 μg/mL) for 24 hours. **(A)** IL-6 and **(B)** TNF-α levels in the culture medium were measured by ELISA and were normalized by the total protein. The results were presented as the percent of LPS control. Each bar represents the mean ± SEM of the three independent experiments. Statistical significance relative to the LPS group was indicated, **$P<0.01$.

Figure 4. 4a11 and **4a16** attenuate the LPS-induced ALI in rats. Rats were intratracheal instillation of LPS. 6 hours later, rats were anaesthetized and killed. Bronchoalveolar lavage fluid and lung tissues were collected for further tests. **(A)** Wet/Dry ratio. **(B)** Protein concentration in BALF. **(C)** HE stain.

Figure 5. 4a11 and **4a16** attenuate the LPS-induced lung inflammation in rats. Rats were intratracheal instillation of LPS. 6 hours later, rats were anaesthetized and killed. Bronchoalveolar lavage fluid and lung tissues were collected for further tests. **(A)** The number of neutrophils in BALF. **(B)** The amount of TNF-α in BALF. **(C)** Immunohistochemical of CD68 stain.

Figure 6. 4a11 and **4a16** inhibited the inflammatory genes expression induced by LPS in Beas-2B. Cells were plated at a density of 7.0×10^5/plate overnight in 37°C and 5% CO_2. Beas-2B were pretreated with 10 μM **4b2** for 30 minutes and subsequently incubated with LPS (1 μg/mL) for 24 hours. Cells were collected and the total RNA was extracted. The mRNA levels of inflammatory cytokines were detected by QPCR **(A-D)**. The results were presented as the percent of LPS control. Each bar represents the mean ± SEM of the three independent experiments. Statistical significance relative to the LPS group was indicated, *P<0.05; **P<0.01.