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Synthesis of gallic acid based naphthophenone fatty acid amides as cathepsin D inhibitors☆

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Abstract—Gallic acid, one of the most abundant plant phenolic acids, has been modified to cathepsin D protease inhibitors. The strategy of modification was proposed basing on some previously reported structure and activity relationship (SAR) studies. The synthesized naphthophenone fatty acid amide derivatives have been evaluated for in vitro cathepsin D inhibition activity. Two of them have shown significant inhibition activity with IC_{50} values of 0.06 and 0.14 μM, respectively, as compared against pepstatin (0.0023 μM), the most potent inhibitor known so far. The study revealed that such attempts on gallic acid based pharmacophores might result in potent inhibitors of cathepsin D.

Cathepsin D (EC 3.4.23.5),[1] a lysosomal aspartic protease enzyme,[2] is ubiquitously expressed in mammals and aves.[3a,3b] Cathepsin D is overproduced and hypersecreted by the cancer cells of breast[4a–e] and ovary.[4e,5] It plays a role in tumour metastasis[6a–e] and also in the proteolysis of the amyloid precursor protein in neurons, a key element of Alzheimer's disease pathology.[7a–c]

Among the reported inhibitors of cathepsin D, pepstatin **1** (an N-acylated pentapeptide) is the most potent one obtained from culture filtrates of actnomycetes.[8a–c] Pepstatin has provided a useful peptidomimetic lead for drug discovery efforts. Besides, there are also several non-peptidic small molecule inhibitors **2**–**5** of cathepsin D[9a–d] with very good IC_{50} values as shown in Figure 1.

Protease inhibitors have been traditionally developed from natural product screening for lead molecules with subsequent optimization. Peptidic inhibitors are less preferred due to their instability, low bioavailability and poor pharmacological profile. To be an effective drug, protease inhibitor needs to have minimal peptidic character, high stability to non-selective proteolytic degradation, good membrane permeability, good bioavailability and long lifetimes in the bloodstream and in cells.

Usually compounds with low molecular weights (<1000 Da) show this type of properties.[10] Recently, it has been convincingly demonstrated that a wide range of proteases like aspartic, serine, cysteine and metallo universally bind to the inhibitors/substrates at their extended β-strand, i.e., the peptidic backbone or equivalent drawn out in linear arrangement.[11a,b] The non-selectivity of these proteases towards substrates was due to their structural flexibility. Therefore, development of a more selective and potent protease inhibitor may lie at the use of more conformationally restricted molecules that fix into a particular protease binding conformation. In this regard, Whitesitt et al.[9a] demonstrated that a benzophenone moiety with a hydroxyl group at 2-position **3** was a better pharmacophore for such type of inhibitors. Considering the features of 2-*O*-naphthyl group of **2** and the hydrophobic pharmacophore of **3**, we speculated a naphthophenone ring with a non-peptidic linear fatty acid at 2-*O*-position as a basic unit to obtain a potent protease inhibitor. Thus, we designed a gallic acid based conformationally restricted naphthophenone moiety **9** (Fig. 2) as a basic unit along with different linear non-peptidic amide side chains at 2-*O*-position. For our study, gallic acid **6** was chosen as a building block for the synthesis of this type of inhibitors due to its natural abundance and common presence in molecules like combretastatin A4,

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Keywords: Synthesis; Gallic acid; Protease inhibitors; Cathepsin-D; Amides.

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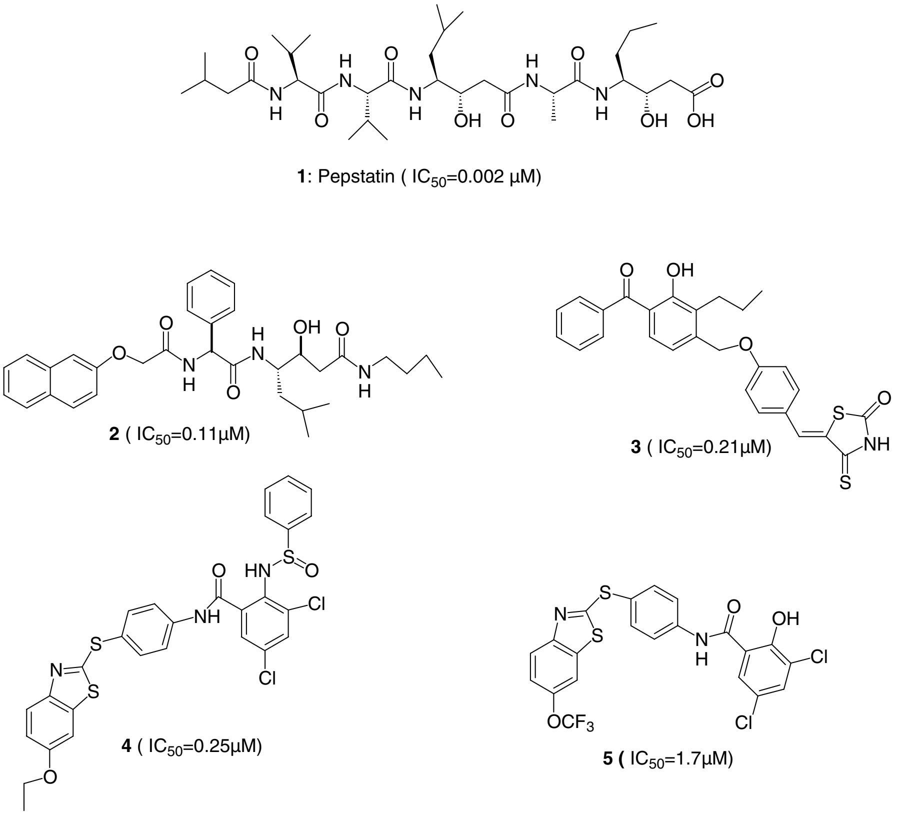


1: Pepstatin (IC_{50}=0.002 µM)

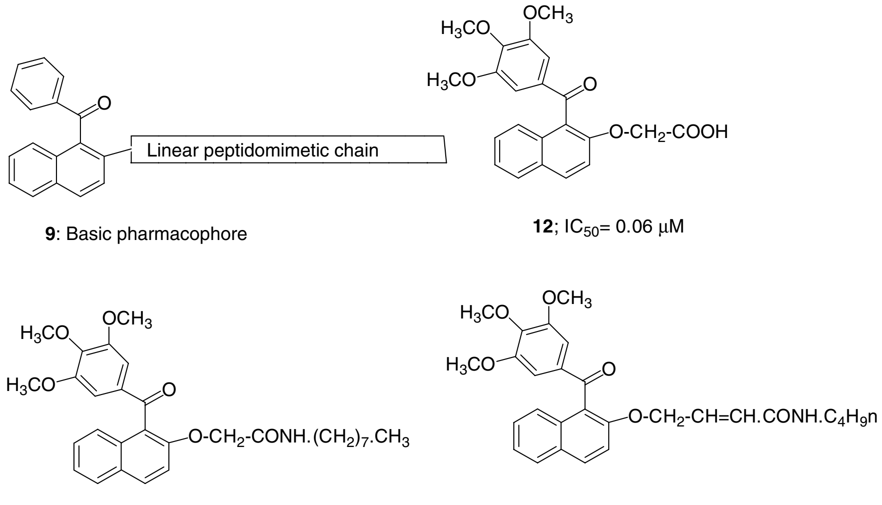
2 (IC_{50}=0.11µM)

3 (IC_{50}=0.21µM)

4 (IC_{50}=0.25µM)

5 (IC_{50}=1.7µM)

Figure 1. Some potent inhibitors of cathepsin D along with their IC_{50} values.



9: Basic pharmacophore

12; IC_{50}= 0.06 µM

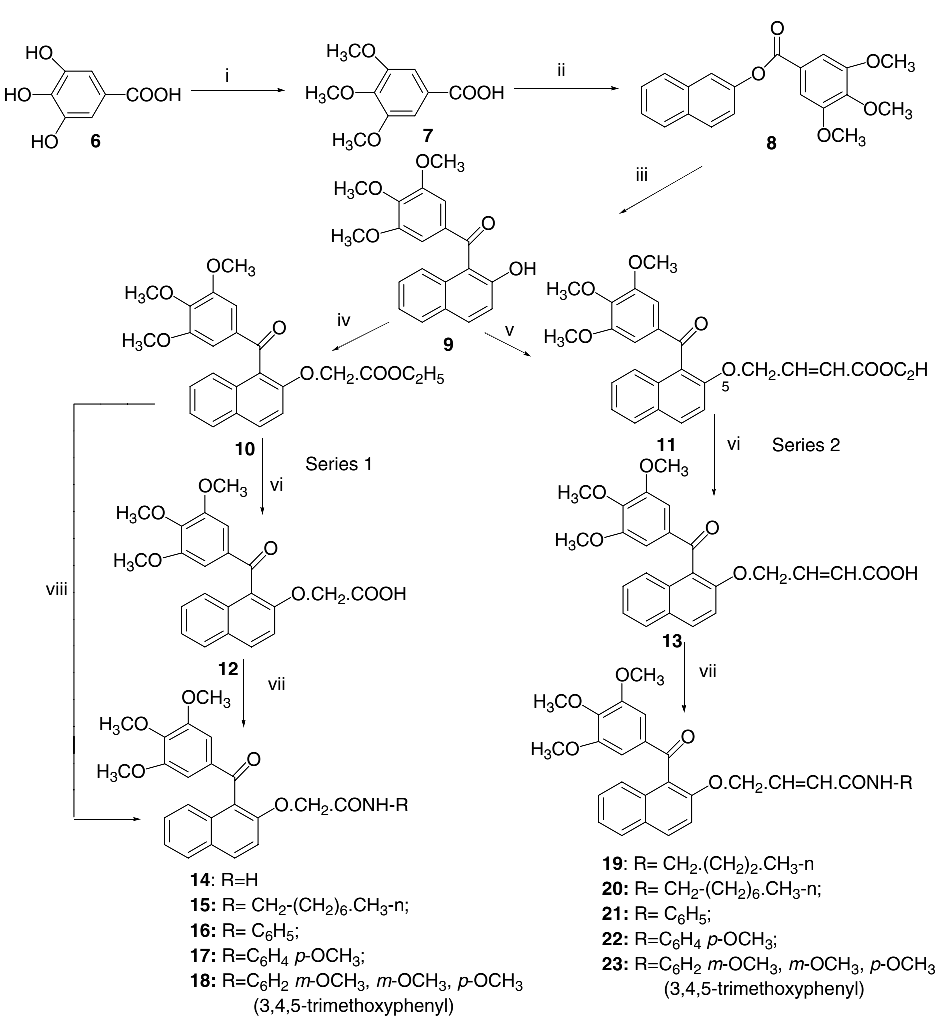
15; IC_{50}= 8.9 µM

19; IC_{50}= 0.14 µM

Figure 2. Gallic acid based pharmacophore and their potent inhibitors of cathepsin D.

podophyllotoxin, etc.[12] Thus, in this communication we report synthesis of different novel derivatives of gallic acid and their human cathepsin-D enzyme inhibition activity. Figure 2 shows three potent inhibitors amongst the various obtained derivatives.

The synthetic strategy adopted for these amides is presented in Scheme 1. To synthesize the targeted basic naphthophenone pharmacophore **9**,[13] gallic acid **6** was methylated with dimethyl sulfate in aqueous potassium hydroxide (20% w/v) to give trimethoxy benzoic acid **7** in 62% yield. Later, the methylated gallic acid **7** was esterified with 2-naphthol in presence of DCC/DMAP in dry dichloromethane under refluxing condition to give 3,4,5-trimethoxy benzoic acid naphthalene-2-yl-ester **8** in 84% yield. Compound **8** was subjected to Fries rearrangement using $AlCl_3$ under neat reaction conditions at 120–130 °C which gave the desired naphthophenone moiety **9** with 31% yield along with two byproducts, 4-demethylated and 3,4-didemethylated



Scheme 1. Reagents and conditions: (i) Me_2SO_4, 20% aq KOH, 10–20 °C for 30 min, then reflux for 3 h, 62%; (ii) DCC, DMAP, DCM, 2-naphthol, reflux, 4 h, 84%; (iii) $AlCl_3$, neat, 120–130 °C, 31%; (iv) ethyl bromoacetate, K_2CO_3, acetone, rt, stir, 2 h, 89%; (v) ethyl bromocrotonate, K_2CO_3, acetone, rt, stir, 3 h, 83%; (vi) 10% aq methanolic (1:1) KOH, 50 °C, 2 h, 84%; (vii) different amines (*n*-butyl amine, *n*-octyl amine, aniline, *p*-anisidine, 3,4,5-trimethoxy aniline except for ammonia), EDC-HCl, HOBt, DMAP, DCM, reflux, 2–5 h, 38–62%; (viii) For **14**: **10** in 30% aqueous ammonia, left overnight at rt, 58%.

derivatives, of starting ester. The Friedel–Crafts reaction on trimethoxy benzoic acid **7** with 2-naphthol in the presence of various Lewis acids ($AlCl_3$-DCM, $AlCl_3$-CS_2, $AlCl_3$ neat, $ZnCl_2$, BF_3-etherate and $SnCl_4$) to get **9** was unsuccessful. However, the reaction was successful only when polyphosphoric acid (PPA) was used, but the yield was less (<5%).

After synthesizing the basic naphthophenone pharmacophore **9**, two linear fatty acid ester chains were hooked at its 2-*O*-position by stirring it at room temperature with two different ethyl bromo esters (ethyl bromo acetate and ethyl bromo crotonate) in anhydrous potassium carbonate and acetone to get desired products **10** and **11**, respectively, in 89% and 83% yields. The ethyl ester derivatives thus obtained were saponified with 10% aqueous methanolic (1:1) potassium hydroxide by heating at 50 °C for two hours. Finally, the desired amides **15**–**23** were obtained after refluxing two acid derivatives **12** and **13** with *N*′-(3-dimethylaminopropyl-*N*-ethyl carbodiimide hydrochloride (EDC-HCl), 1-hydroxy benzotriazole (HOBt) and DMAP in dry dichloromethane for 2–4 h in 48–82% yields.[15] However, the amide **14** was obtained in 58% yield while leaving compound **10** through overnight in 30% aqueous ammonia at room temperature. The spectral data of selected compounds is also given.[17]

All the amide derivatives were evaluated for in vitro cathepsin-D inhibition activity as per reported method.[14,16] Bovine haemoglobin and cathepsin-D from human liver were used as substrate and enzyme, respectively. Both the substrate and enzyme were procured from Sigma. All the statistical calculations of biological assays were computed using ‘Graph pad prism’ software. Three different experiments were conducted to find the enzyme kinetics, inhibition of enzyme activity by pepstatin and inhibition of enzyme activity by the test compounds **10** and **12**–**23**. In the enzyme activity assay the kinetic parameters of cathepsin D activity were found to be $V_{max} = 0.0051$ and $K_m = 0.0085$. The IC_{50} values were calculated by using Michaelis–Menten equation and are given in Table 1.

Table 1. IC_{50} values of pepstatin A and test compounds against cathepsin D enzyme activity

S. no.	Compound	IC_{50} (μM)
1	Pepstatin A	0.0023
2	**10**	Inactive
3	**12**	0.06
4	**13**	Inactive
5	**14**	n.d.
6	**15**	8.9
8	**16**	231.5
9	**17**	Inactive
10	**18**	n.d.
11	**19**	0.14
12	**20**	n.d.
13	**21**	Inactive
14	**22**	4.71
15	**23**	12.29

n.d., not determined.

The IC_{50} of pepstatin was found to be 0.0023 μM. Two of the test compounds **12** and **19** showed high level of inhibition (IC_{50}; 0.06 and 0.14 μM), whereas **15** and **22**–**23** showed moderate level of inhibition. Compound **16** had shown low activity of IC_{50} value at 231.5 μM. Rest were inactive as their IC_{50} values were higher than 1000 μM.

The amides derived from aliphatic amines showed better activities as compared to those derived from aromatic amines in both the cases (Scheme 1, synthesized from compounds **10** and **11**). However, the amides derived from aromatic amines in the crotonate chain (synthesized from **11**) possessed better activity than the aromatic amides with acetate chain (synthesized from **10**) probably due to the presence of crotonate (four carbon) chain at 2-*O* position. Further, the low activity of aromatic amides when compared to aliphatic ones might be due to the bulky aryl moiety, which is altering the ligand–receptor interaction in the enzyme pocket. Thus, it may be concluded that pharmacophores like gallic acid based naphthophenone systems with linear aliphatic chain attached at amide nitrogen will be better inhibitors for cathepsin D enzyme.

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15. Syntheses. (a) General procedure for the synthesis of **12** and **13**. Synthesis of 2-[1-(3,4,5-trimethoxybenzoyl) naphthalene-2-yloxy] ethanoic acid **12**: In a 25 ml round-bottomed flask 2-[1-(3,4,5-trimethoxy-benzoyl)-naphthalen-2-yloxy]-ethyl ethanoate **10** (100 mg, 0.23 mmol) was taken in 5 ml of 10% aqueous methanolic (1:1, v/v) potassium hydroxide. The reaction mixture was stirred at 50 °C for 2 h. On completion, the reaction mixture was acidified with 10% HCl and extracted with ethyl acetate

(3× 20 ml). The organic layer was washed with water and concentrated to get **12** as white crystalline solid. (b) Synthesis of 2-[3,4,5-trimethoxybenzoyl naphthalene-2-yloxy]-ethanoic acid amide **14**: In a round-bottomed flask 2-[1-(3,4,5-trimethoxy-benzoyl) naphthalene-2-yloxy]-ethyl ethanoate **10** (100 mg, 0.25 mmol) was taken in 5 ml aqueous ammonia (30%). The reaction mixture was kept as such for overnight at room temperature. Later the reaction mixture was diluted with water and extracted with ethyl acetate (3× 25 ml). The organic layer was washed with water, dried over anhydrous sodium sulfate and distilled off to get a creamy white solid compound **14**. (c) General procedure for the synthesis of amide derivatives **15**–**23**. Synthesis of 4-[1-(3,4,5-trimethoxybenzoyl) naphthalene-2-yloxy]-but-2-enoic acid-3,4,5-trimethoxy phenyl amide **23**: In a 25 ml round-bottomed flask 4-[1-(3,4,5-trimethoxy-benzoyl)-naphthalen-2-yloxy]-but-2-enoic acid **10** (100 mg, 0.171 mmol) was taken in dry dichloromethane (10 ml). To this, EDC (41 mg, 0.21 mmol), HOBt (29 mg, 0.21 mmol) and DMAP (5 mg) were added and stirred at room temperature for 20 min. Later 3,4,5-trimethoxy aniline (46 mg, 0.25 mmol) was added to the reaction mixture and refluxed for 3 h. On completion, water (10 ml) was added to it and extracted with chloroform (3× 20 ml). The organic layer was washed with water, dried over anhydrous sodium sulfate and evaporated. The residue thus obtained was purified through a silica gel column by eluting with hexane–ethyl acetate. The desired amide was obtained at 5% ethyl acetate–hexane fraction, which on concentration yielded a creamy white solid compound.

16. Biological evaluation. Cathepsin D inhibition assay: The enzyme inhibition assay was performed using spectrophotometric stop rate determination method. A reaction cocktail containing haemoglobin solution (10 ml, 2.5% w/v), citrate buffer (400 mM, pH 2.8, 2.5 ml) and deionised water (10 ml) was freshly prepared. The pH of the contents was adjusted to 3.0 by using dil HCl. To the 2.24 ml of reaction cocktail, 0.01 ml of different concentrations (1000, 100, 10 and 1 µg/ml, in DMSO) of test samples (pepstatin and compounds **10**–**23**) was added, mixed well and left for 30 min. A blank sample was prepared by adding 0.01 ml DMSO into 2.24 ml of reaction cocktail. To the reaction mixture (except blank where enzyme was added after adding TCA), 0.01 ml enzyme solution (0.1 µg/ml) was added and incubated for 10 min at 37 °C. After this, 2.25 ml (5%, v/v) trichloroacetic acid was added to stop the enzymatic reaction and the contents were shaken well and filtered through 0.45 µM syringe filter. The supernatant was taken and read spectrophotometrically at 280 nm. The enzyme activity was calculated by the formula

$$\text{Units/ml enzyme activity} = \frac{(A_{280\text{nm}}\text{Test} - A_{280\text{nm}}\text{Blank})(4.51)(\text{df})}{(10)(0.01)(1)},$$

where 4.51 = total volume of assay (in ml), df = dilution factor, 10 = time of assay (in minutes), 0.01 = volume of enzyme (in ml), 1 = increase in 280 nm per minute as per the unit definition. The IC_{50} values were calculated from this enzyme activity value for each test compound by using Michaelis–Menten equation in 'Graph pad prism' software.

17. Selected physical data: Compound **12**: Yield: 78%; mp 132–135 °C; IR (KBr, cm^{-1}): 3451 (OH, acid), 1713 (acid carbonyl), 1659 (ketone), 1585 and 1506 (aromatic), 1249 and 1127 (ether); ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.75 (6H, s, 3,5-OC*H*$_3$), 3.92 (3H, s, 4-OC*H*$_3$), 4.77 (2H, s, OC*H*$_2$), 6.2 (1H, br s, exchangeable, COO*H*), 7.16–7.96 (8H, m, all aromatic protons); ^{13}C NMR (75 MHz, $CDCl_3$): δ 56.73, 56.73, 60.86, 67.17, 108.94, 108.94, 114.66, 124.94, 124.94, 125.11, 126.06, 127.70, 128.30, 129.97, 131.77, 132.15, 133.33, 150.67, 152.60, 153.68, 170.39, 196.11. Electrospray mass (CH_3CN): 397 $[M+H]^+$, 419 $[M+Na]^+$, 435 $[M+K]^+$. Elemental Anal. Calcd for $C_{22}H_{20}O_7$: C, 66.66; H, 5.09. Obsd: C, 67.14; H, 4.87.
Compound **13**: Yield: 69%; mp 132–35 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.87 (6H, s, 3,5-OC*H*$_3$), 4.08 (s, 4-OC*H*$_3$), 6.63 (1H, d, =C*H*COOH, *J* = 15.6 Hz), 6.72 (2H, s, OC*H*$_2$), 7.53–7.58 (1H, d, C*H*=, *J* = 15.6 Hz), 7.40–7.96 (8H, m, all aromatic protons); ^{13}C NMR (75 MHz, $CDCl_3$): δ 14.14, 56.55, 56.55, 60.38, 60.81, 67.02, 108.0, 108.0, 114.56, 122.45, 124.58, 124.67, 127.57, 128.14, 129.54, 131.16, 131.30, 132.16, 148.13, 152.83, 153.52, 165.73, 194.61. Electrospray mass: 445 $[M+Na]^+$. Elemental Anal. Calcd for $C_{24}H_{22}O_7$: C, 68.24; H, 5.25. Obsd: C, 68.46; H, 5.48.
Compound **14**: Yield: 48%, mp 107–109 °C, ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.75 (6H, s, 3 and 5 OC*H*$_3$), 3.92 (3H, s, 4-OC*H*$_3$), 5.44 and 6.60 (2H, br s, N*H*$_2$), 4.63 (2H, s, OC*H*$_2$), 7.11–8.01 (8H, m, all the aromatic protons). Electrospray mass (CH_3CN): 418$[M+Na]^+$. Elemental Anal. Calcd for $C_{22}H_{21}NO_6$: C, 66.83; H, 5.35; N, 3.54. Obsd: C, 67.28; H, 5.07; N, 3.22.
Compound **15**: Yield: 62%, mp oil, ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.85 (3H, br s, C*H*$_3$), 1.25–1.28 (12H, br s, 6× C*H*$_2$), 3.11–3.16 (2H, br s, NC*H*$_2$), 3.70 (6H, s, 3,5-OC*H*$_3$), 3.93 (3H, s, 4-OC*H*$_3$), 6.43 (1H, bs, N*H*), 4.58 (2H, s, OC*H*$_2$), 7.13–7.72 (8H, m, all the aromatic protons). Electrospray mass (CH_3CN): 508 $[M+H]^+$. Elemental Anal. Calcd for $C_{30}H_{37}NO_6$: C, 70.98; H, 7.35; N, 2.76. Obsd: C, 70.85; H, 7.42; N, 3.04.
Compound **17**: Yield: 62%, mp 143–145 °C, ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.79 (6H, s, 3,5-OC*H*$_3$),3.83 (3H, s, OC*H*$_3$ of *p*-anisidine ring), 3.91 (3H, s, 4-OC*H*$_3$), 4.81 (2H, s, OC*H*$_2$), 6.83–7.94 (12H, m, all the aromatic protons). Electrospray mass (CH_3CN): 502 $[M+H]^+$. Elemental Anal. Calcd for $C_{29}H_{27}NO_7$: C, 69.45; H, 5.43; N, 2.79. Obsd: C, 69.22; H,5.83; N, 3.16.
Compound **18**: Yield: 38%, mp 152–155 °C, ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.74 (3H, s, OC*H*$_3$), 3.78 (6H, s, 2× OC*H*$_3$), 3.81 (6H, s, 2× OC*H*$_3$), 3.90 (3H, s, 4-OC*H*$_3$), 4.89 (2H, s, OC*H*$_2$), 6.68–7.89 (10H, m, all the aromatic protons). Electrospray mass (CH_3CN): 562 $[M+H]^+$. Elemental Anal. Calcd for $C_{31}H_{31}NO_9$: C, 66.33; H, 5.56; N, 2.49. Obsd: C, 65.92; H, 5.23; N, 2.94.
Compound **19**: Yield: 57%, mp oil, ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.85 (3H, t, C*H*$_3$, *J* = 7.22 Hz), 1.32 (4H, m, C*H*$_2$-C*H*$_2$), 3.30 (2H, br d, N–C*H*$_2$), 3.77 (3H, s, OC*H*$_3$), 3.78 (6H, s, 3,5-OC*H*$_3$), 3.91 (3H, s, OC*H*$_3$), 6.52 (1H, d, =C*H*, *J* = 15.2 Hz), 6.62 (2H, s, OC*H*$_2$), 6.63–7.75 (9H, m, all the aromatic protons and C*H*=). Electrospray mass (CH_3CN): 478 $[M+H]^+$. Elemental Anal. Calcd for $C_{28}H_{31}NO_6$: C, 70.42; H, 6.54; N, 2.93. Obsd: C, 69.92; H, 6.23; N, 3.34.
Compound **22**: Yield: 46%, mp 127–129 °C, ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.78 (3H, s, OC*H*$_3$), 3.84 (6H, s, 3,5-OC*H*$_3$), 3.97 (3H, s, 4-OC*H*$_3$), 6.69 (2H, s, OC*H*$_2$), 6.72 (1H, d, =C*H*, *J* = 15 Hz), 6.85–7.84 (13H, m, all the aromatic protons and C*H*=). Electrospray mass (CH_3CN): 528 $[M+H]^+$. Elemental Anal. Calcd for $C_{31}H_{29}NO_7$: C, 70.58; H, 5.54; N, 2.65. Obsd: C, 70.66; H, 5.92; N, 3.09.

Compound **23**: Yield: 64 mg (46%), mp 172–175 °C, ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.85 (15H, s, 5× OCH_3, 3,5-OCH_3 and 3,4,5-OCH_3), 4.01 (3H, s, 4-OCH_3), 6.72 (2H, br s, OCH_2), 6.99–7.94 (10H, m, all the aromatic protons and $=CH$ and $CH=$). Electrospray mass (CH_3CN): 588 $[M+H]^+$, 610 $[M+Na]^+$. Elemental Anal. Calcd for $C_{33}H_{33}NO_9$: C, 67.45; H, 5.66; N, 2.38. Obsd: C, 67.77; H, 6.04; N, 2.97.