Synthesis and Peptidyl-Prolyl Isomerase Inhibitory Activity of Quinoxalines as Ligands of Cyclophilin A

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In search of small molecule compounds as the ligands of cyclophilin A, a series of quinoxalines were prepared, and their K_d values of cyclophilin A and IC_{50} values for peptidyl-prolyl isomerase activity of cyclophilin A were tested. The results suggest that some quinoxalines are promising ligands of cyclophilin A.

Key words quinoxaline; peptidyl-prolyl isomerase inhibitory activity; cyclophilin A; ligand

Cyclophilins comprise a superfamily of ubiquitously expressed cellular proteins with peptidyl-prolyl isomerase (PPIase) activity,[1,2] and are supposed to play roles in diverse cellular processes.[3] Cyclophilin A (CyPA) is the first characterized isoform of cyclophilins, and an abundant cytosolic isoform as the major target of cyclosporin A (CsA).[1,4—6] CyPA with PPIase activity is a cellular enzyme that plays important roles in cellular signaling and protein folding.[1,2,7] CsA binds CyPA, leading to inhibition of calcineurin, which dephosphorylates the apoptogenic protein Bad and enables it to bind other antiapoptotic Bcl-2 family members, and trigger release of cytochrome C and other proapoptotic proteins from the intermembrane space.[8] The experimental evidence has been provided for efficient CyPA catalysis on a natively folded and biologically relevant protein substrate.[9—11]

The process of protein profiling has the potential to expand the current menu of molecular targets. This type of information should lead to more rationally designed diagnostic and treatment strategies.[12]

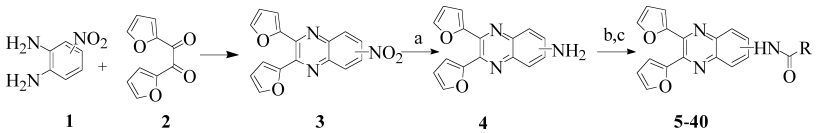
Almost all ligands of cyclophilins are peptides. In recent years, a number of non-peptidic small molecule ligands have been found with diverse activities.[13—15]

Quinoxalines used to be antagonists of NMDA receptor,[16,17] central serotonin activation agents[18] and herbicide.[19] Recently, we found that quinoxaline derivatives could be the ligands of CyPA by means of virtual screening. In this paper, a series of derivatives of quinoxaline are reported as the new ligands of human cyclophilin A (hCyPA).

Compounds **5**—**40** were prepared as shown in Chart 1. 2,3-Bis(furan-2-yl)-5 or 6-nitroquinoxaline (**3**) were obtained by condensation of 3- or 4-nitro-*o*-phenylenediamine (**1**) with furil (**2**). Reduction of nitro compound **3** by hydrogenation under catalysis of Pd/C afforded amines **4**. The quinoxalines **5**—**40** were finally yielded by the reaction of 5- or 6-amino-2,3-bis(furan-2-yl)quinoxaline (**4**), triethylamine and triphosgene, followed by the addition of the corresponding amines or alcohols. The crude products were purified by flash column chromatography over silica gel to give yellow or brown amorphous solid, which are unstable, and decomposed on heating.

Virtual Screening Based on the crystal structure of cyclophilin A (PDB code: 1NMK), we searched the database of the Chinese National Center for Drug Screening which contains more than 11700 compounds by virtual screening. The molecular docking program DOCK4.0 was employed to do the primary screening. In the database search, conformational flexibility of the small molecules was taken into account. The small molecules were ranked according to their scores calculated by the energy scoring function in the DOCK program. The top 500 candidate molecules with the best scores were kept for next step. The Cscore was employed to re-evaluate the DOCK 500 molecules and 100 compounds with Cscore better than 4 were kept. The 100 compounds were subjected to further screening by using FlexX. Thirty-two compounds with FlexX energy score less than −30.0 kcal/mol were put aside for further studies. Finally, 10 compounds were selected with visual inspection for biological assay. Among them 6 compounds showed the binding affinity to cyclophilin A at micro-molar level.

Surface Plasmon Resonance Technology-Based Biacore 3000 Analyses[20] The interactions between compounds **5**—**40** and CyPA were performed using the dual flow cell Biacore 3000 instrument (Biacore AB, Uppsala, Sweden). All the experiments were carried out using HBS-EP (10 mmol/l *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 150 mmol/l NaCl, 3.4 mmol/l EDTA and 0.005% surfactant P20 at pH 7.4) as a running buffer at a constant flow rate of 20 μl/min at 25 °C. The protein was immobilized directly and covalently on the hydrophilic carboxymethylated dextran matrix of the CM5 sensor chip (BIAcore) by using the standard primary amine coupling reaction. The protein to be bound to the sensor chip was diluted in 10 mmol/l sodium acetate buffer (pH 6.5) to a concentration of 17 μmol/l. The concentrations of the compounds dissolved in the running



Reagents: (a) H_2, 5% Pd/C, EtOH. (b) $(CCl_3O)_2CO$, TEA, CH_2Cl_2. (c) Amine or alcohol.

Chart 1

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Table 1. Structures of Compounds and K_d Values of the Complexes of hCyPA-Ligand[a)]

Compounds	Position of amino	R	K_d (μM)
5	6	OEt	>100
6	6	OAm^i	3.58
7	6	NEt_2	>100
8	6	NPr^i_2	59.8
9	6	NBu^n_2	15.1
10	6	NBu^i_2	>100
11	6	$NHPr^i$	>100
12	6	$MeNBu^n$	>100
13	6		>100
14	6		6.79
15	6		>100
16	6		7.56
17	6		>100
18	6		3.74
19	6		1.86
20	6		37.5
21	6		1.72
22	6		2.20
23	6		nd[b)]
24	6		8.79
25	6		1.88
26	6		7.58
27	6		2.52
28	6		6.46
29	6	$NHCH_2CONEt_2$	5.12
30	6	$NH(CH_2)_2CONEt_2$	2.73
31	6	$NH(CH_2)_5CONEt_2$	4.15
32	6	$NHCH_2CO_2Et$	59.4
33	6		>100
34	6		>100
35	6		>100
36	6		>100
37	6		>100
38	5		>100
39	5		5.54
40	5	OAm^i	>100

a) The K_d was measured by SPR based Biacore 3000 with the procedure of Luo *et al.*[20)] hCyPA was obtained according to the procedure in ref. 20. *b*) nd: not detected.

buffer varied from 1.18 to 10 μmol/l. All the data analyses were carried out using BIAevaluation software, and the sensorgrams were processed by automatic correction for nonspecific bulk refractive index effects. The kinetic analyses of the ligand binding to the protein were performed based on the 1 : 1 Langmuir binding fit model according to the procedures described in the software manual.

PPIase Inhibition Activity Assay The PPIase activity assay for the CyPA was performed based on a published method[21)] with some modifications. The substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanlilide (Suc-AAPF-pNA, S-7388) and α-chymotrypsin (C-7762) were purchased from Sigma (St. Louis, Missouri, U.S.A.). Suc-AAPFpNA was dissolved in tetrahydrofuran containing 400 mmol/l of LiCl, and the stock solution concentration was 10 mmol/l. α-chymotrypsin was dissolved in 1 mmol/l HCl containing 2 mmol/l $CaCl_2$, and the stock solution concentration was 80 mmol/l. The assay buffer (173 μl of 50 mmol/l HEPES, 100 mmol/l NaCl, pH 8.0 at 0 °C. final concentration 43 mmol/l HEPES, 86 mmol/l NaCl), 15 μl of de-ionized water and CyPA (2 μl of a 2.7 μmol/l stock solution) and the compounds (final concentration ranging from 100 nmol/l to 50 μmol/l) were pre-equilibrated for 3 h on ice. Immediately before the assay was started, 7.5 μl of chymotrypsin solution was added. Absorbance readings at 390 nm were recorded when 2.5 μl of the peptide substrate was added into the 1 cm path length cuvette and the solution was mixed rapidly. The data were collected on a Hitachi U2010 spectrophotometer.

Results and Discussion

By using surface plasmon resonance (SPR) method, the kinetic analysis of the hCyPA/ligand interaction of compounds **5**—**40** was investigated (Table 1). Based on the K_d values, 17 compounds (**6**, **9**, **14**, **16**, **18**, **19**, **21**, **22**, **24**—**31**, **39**) displayed a moderate binding affinity to hCyPA in the micromolar concentration range. Among them, the substituted carbamoyl piperidino and carbamoyl pyrrolidino containing compounds **19**, **21** and **25** showed the highest affinity, and compounds **8**, **20** and **32** showed lower binding affinity. When R is simple alkoxy (**5**, **40**), alkylamino (**7**, **10**—**12**), simple cyclic amino (**13**, **15**, **17**) and bulky amino acid moiety (**33**—**37**), these quinoxalines did not show any affinity (K_d>100 μM).

In comparison of the activity of two enantiomers **18** and **19**, they displayed the affinity to hCyPA at the same level of concentration and so did the enantiomers **25** and **26**. It seems that both enantiomers of the chiral quinoxalines are responsible for the affinity to hCyPA.

The IC_{50} values of some quinoxalines for PPIase activity of hCyPA were also measured (Table 2). The K_d and IC_{50} values of the compounds in Tables 1 and 2 are not parallel. There is no simple correlation between K_d and IC_{50} values of these compounds. Compounds **14** and **29** showed distinct inhibition against PPIase activity of hCyPA. However, they are 5-fold less potent than CsA.

The data in Tables 1 and 2 demonstrate that quinoxaline is a new type of the ligand of cyclophilin A and a few of quinoxaline compounds are the inhibitors against PPIase activity of hCyPA.

Table 2. IC_{50} Values of Some Quinoxalines for PPIase Activity of hCyPA[a)]

Compounds	IC_{50}[b)] (μM)
CsA	0.0614
6	0.814
14	0.347
23	0.683
24	0.534
25	1.00
28	0.451
29	0.312
30	3.18
31	0.924

a) The PPIase inhibitory activity was measured by the standard chymotrypsin-coupled assay with the procedure of Kofron *et al.*[21)] *b*) Values are means of three different experiments.

Experimental

Infrared (IR) spectra were measured with a NicoletMagna IR750 spectrophotometer. ^{1}H- (400 MHz) and ^{13}C-NMR (100 MHz) spectra were recorded in chloroform-*d* or DMSO-d_6 on a Bruker AMX-400 and Gemini-300 spectrometer. Mass spectra were taken on a Varian MAT-711 and MAT-95 mass spectrophotometer.

Preparation Procedure of 6-Aminoquinoxalines 6-Nitro-2,3-di(furan-2-yl)quinoxaline (**3a**): A mixture of 4-nitrobenzene-1,2-diamine (7.6 g, 50 mmol) and furil (9.5 g, 50 mmol) in ethanol (200 ml) was heated at reflux for 20 h. Then the mixture was cooled with ice bath and filtered to give crude product. Recrystallization from nitromethane gave **3a** (11 g, 72%) as a golden needle crystal. ^{1}H-NMR ($CDCl_3$) δ: 6.63 (2H, m), 6.87 (2H, m), 7.68 (2H, m), 8.24 (1H, d, J=9.2 Hz), 8.50 (1H, dd, J=2.7, 9.1 Hz), 9.01 (1H, d, J=2.5 Hz).

6-Amino-2,3-di(furan-2-yl)quinoxaline (**4a**): 6-Nitro-2,3-di(furan-2-yl)-quinoxaline (**3a**, 1.6 g, 5.2 mmol) in ethanol (20 ml) was hydrogenated in the presence of 5% Pd/C (50 mg) under 1 atm at room temperature for 3 h. The mixture was filtered. The solid was treated with DMSO (2 ml) and the mixture was filtered through a pad of celite. The filtrate was poured into water (200 ml), and the precipitate was filtered to give **4a** (1.2 g, 85%) as a yellow solid. ^{1}H-NMR ($CDCl_3$) δ: 6.55 (2H, m), 6.61 (1H, d, J=3.4 Hz), 6.69 (1H, d, J=3.5 Hz), 7.21 (1H, dd, J=2.5, 9.0 Hz), 7.41 (1H, d, J=2.4 Hz), 7.58 (1H, dd, J=0.3, 1.3 Hz), 7.63 (1H, d, J=1.7 Hz),7.91 (1H, d, J=8.9 Hz).

Preparation Procedure of 5-Aminoquinoxalines 5-Nitro-2,3-di(furan-2-yl)quinoxaline (**3b**): A mixture of 3-nitrobenzene-1,2-diamine (1.53 g, 10 mmol) and furil (1.9 g, 10 mmol) in toluene (40 ml) was heated at reflux for 20 h. Then the mixture was cooled with ice bath and filtered to give crude product. Recrystallization from nitromethane gave **3b** (2.2 g, 72%) as a yellow needle crystal. ^{1}H-NMR ($CDCl_3$) δ: 6.60 (2H, m), 6.79 (1H, dd, J=0.7, 3.4 Hz), 7.06 (1H, dd, J=0.7, 3.5 Hz), 7.59—7.80 (3H, m), 8.14 (1H, dd, J=1.4, 7.6 Hz), 8.32 (1H, dd, J=1.4, 8.5 Hz).

5-Amino-2,3-di(furan-2-yl)quinoxaline (**4b**): 5-Nitro-2,3-di(furan-2-yl)-quinoxaline (**3b**, 1.0 g, 3.6 mmol) in ethanol (40 ml) was hydrogenated in the presence of 5% Pd/C (80 mg) under 1 atm at room temperature for 3 h. The mixture was filtered. The solid was treated with DMSO (2 ml) and the mixture was filtered through a pad of celite. The filtrate was poured into water (200 ml), and the precipitate was filtered to give **4b** (0.86 g, 95%) as a yellow solid. ^{1}H-NMR ($CDCl_3$) δ: 6.52—6.57 (3H, m), 6.72 (1H, dd, J=0.9, 3.5 Hz), 6.90 (1H, dd, J=1.2, 7.6 Hz), 7.45—7.61 (4H, m).

General Preparation Procedure for Quinoxalines 5—40 Ethyl 2,3-di(furan-2-yl)quinoxalin-6-ylcarbamate (**5**): To a solution of **4a** (83.1 mg, 0.30 mmol) and triethylamine (100 μl, 0.72 mmol) in dichloromethane (10 ml), was added triphosgene (30 mg, 0.10 mmol) while stirring. The mixture was stirred at room temperature for 1 h, then ethanol (100 μl, 1.7 mmol) was added. After stirring for an additional hour, the solvent was evaporated in vacuum to give the crude product, which was further purified by flash column chromatography on a silica gel using petro-ether/ethyl acetate (3 : 1) as the eluent to give the desired compound **5** (45.2 mg, 43% yield) as a yellow amorphous solid. ^{1}H-NMR ($CDCl_3$) δ: 1.32 (3H, t, J=7.0 Hz), 4.28 (2H, q, J=7.0 Hz), 5.54 (2H, m), 6.62 (2H, m), 7.17 (1H, s), 7.60 (2H, m), 7.87 (1H, dd, J=2.2, 9.1 Hz), 8.05 (1H, d, J=9.1 Hz), 8.10 (1H, d, J=2.4 Hz). IR (KBr) cm^{-1}: 3419, 3246, 2982, 2928, 1732, 1622, 1572, 1539, 1495, 1261, 1226. MS *m*/*z*: 349 (M^+), 303. EI-MS *m*/*z*: 349.1068 (Calcd for $C_{19}H_{15}N_3O_4$: 349.1063).

Compound **6**: A brown amorphous solid, yield 57%. ^{1}H-NMR ($CDCl_3$) δ: 0.84 (6H, m), 1.45 (2H, m), 1.60 (1H, m), 4.14 (2H, t, J=6.8 Hz), 6.50 (2H, m), 6.58 (2H, m), 7.54 (2H, m), 7.88—7.97 (3H, m), 8.08 (1H, d, J=2.0 Hz). IR (KBr) cm^{-1}: 3253, 3116, 2956, 1734, 1622, 1572, 1537, 1495, 1425, 1331, 1217. MS *m*/*z*: 391 (M^+), 303. EI-MS *m*/*z*: 391.1517 (Calcd for $C_{22}H_{21}N_3O_4$: 391.1532).

Compound **7**: A brown amorphous solid, yield 56%. ^{1}H-NMR ($CDCl_3$) δ: 1.25 (6H, t, J=7.2 Hz), 3.42 (4H, q, J=7.2 Hz), 6.54 (2H, m), 6.61 (2H, ddd, J=0.7, 3.5, 12.2 Hz), 6.78 (1H, s), 7.59 (2H, dd, J=0.7, 1.0 Hz), 7.96 (1H, d, J=1.0 Hz), 8.03 (2H, d, J=1.8 Hz). IR (KBr) cm^{-1}: 3440, 3114, 2972, 2929, 1649, 1524, 1491, 1429, 1265. MS *m*/*z*: 376 (M^+), 303. EI-MS *m*/*z*: 376.1528 (Calcd for $C_{21}H_{20}N_4O_3$: 376.1535).

Compound **8**: A brown amorphous solid, yield 36%. ^{1}H-NMR ($CDCl_3$) δ: 1.32 (12H, d, J=6.9 Hz), 3.99 (2H, m), 6.51 (2H, m), 6.59 (2H, m), 6.76 (1H, s), 7.90 (1H, d, J=2.3 Hz), 7.99—8.06 (2H, m), 7.57 (2H, m). IR (KBr) cm^{-1}: 3427, 2968, 2928, 1647, 1566, 1520, 1495, 1433, 1375, 1205. MS *m*/*z*: 404 (M^+), 303. EI-MS *m*/*z*: 404.1857 (Calcd for $C_{23}H_{24}N_4O_3$: 404.1848).

Compound **9**: A brown amorphous solid, yield 30%. ^{1}H-NMR ($CDCl_3$) δ: 0.95 (6H, t, J=7.3 Hz), 1.36 (4H, m), 1.60 (4H, m), 3.32 (4H, t, J=7.7 Hz), 6.52 (2H, m), 6.59 (2H, m), 6.79 (1H, s), 7.57 (2H, m), 7.89 (1H, d, J=2.4 Hz), 7.97—8.09 (2H, m). IR (KBr) cm^{-1}: 3406, 2958, 2929, 2872, 1647, 1570, 1524, 1489, 1427, 1381, 1336, 1306, 1256, 1223, 1203. MS *m*/*z*: 432 (M^+), 303. EI-MS *m*/*z*: 432.2158 (Calcd for $C_{25}H_{28}N_4O_3$: 432.2162).

Compound **10**: A brown amorphous solid, yield 25%. ^{1}H-NMR ($CDCl_3$) δ: 0.95 (12H, d, J=6.6 Hz), 2.04 (2H, m), 3.19 (4H, d, J=7.6 Hz), 6.52 (2H, m), 6.60 (2H, m), 6.84 (1H, s), 7.57 (2H, m), 7.89 (1H, d, J=2.3 Hz), 7.99—8.08 (2H, m). IR (KBr) cm^{-1}: 3427, 2958, 2928, 2870, 1643, 1570, 1525, 1483, 1427, 1385, 1336, 1256, 1203. MS *m*/*z*: 432 (M^+), 303. EI-MS *m*/*z*: 432.2149 (Calcd for $C_{25}H_{28}N_4O_3$: 432.2161).

Compound **11**: A brown amorphous solid, yield 28%. ^{1}H-NMR ($CDCl_3$) δ: 1.19 (6H, d, J=6.5 Hz), 3.93 (1H, m), 4.68 (2H, b), 6.56 (4H, b), 7.60 (2H, b), 7.85—8.06 (3H, m). IR (KBr) cm^{-1}: 3423, 2968, 2926, 1639, 1564, 1541, 1493, 1423, 1385, 1334, 1234. MS *m*/*z*: 362 (M^+), 303. EI-MS *m*/*z*: 362.1371 (Calcd for $C_{20}H_{18}N_4O_3$: 362.1379).

Compound **12**: A brown amorphous solid, yield 98%. ^{1}H-NMR ($CDCl_3$) δ: 0.96 (3H, t, J=7.3 Hz), 1.39 (2H, q, J=7.5 Hz), 1.60 (2H, m), 3.06 (3H, s), 3.39 (2H, t, J=7.5 Hz), 6.56 (2H, m), 6.59 (1H, d, J=3.3 Hz), 6.62 (1H, d, J=3.5 Hz), 6.78 (1H, s), 7.59 (2H, m), 7.95 (1H, t, J=1.3 Hz), 8.02 (2H, d, J=1.4 Hz). IR (KBr) cm^{-1}: 3373, 3114, 2956, 2929, 2872, 1651, 1570, 1525, 1481, 1425, 1385, 1329, 1308, 1256, 1225, 1203. MS *m*/*z*: 390 (M^+), 303. EI-MS *m*/*z*: 390.1690 (Calcd for $C_{22}H_{22}N_4O_3$: 390.1692).

Compound **13**: A brown amorphous solid, yield 94%. ^{1}H-NMR ($CDCl_3$) δ: 2.00 (4H, t, J=6.6 Hz), 3.53 (4H, t, J=6.6 Hz), 6.55 (2H, m), 6.60 (1H, dd, J=0.70, 3.4 Hz), 6.65 (1H, d, J=3.4 Hz), 6.68 (1H, s), 7.60 (2H, m), 8.02 (2H, m), 8.18 (1H, dd, J=2.3, 9.2 Hz). IR (KBr) cm^{-1}: 3404, 2970, 2877, 1672, 1568, 1525, 1502, 1429, 1382, 1340, 1203. MS *m*/*z*: 374 (M^+), 303. EI-MS *m*/*z*: 374.1360 (Calcd for $C_{21}H_{18}N_4O_3$: 374.1379).

Compound **14**: A brown amorphous solid, yield 88%. ^{1}H-NMR ($CDCl_3$) δ: 1.60 (4H, b), 1.82 (2H, b), 3.48 (4H, b), 6.53 (2H, m), 6.58 (1H, m), 6.61 (1H, m), 7.00 (1H, b), 7.57 (2H, m), 7.90—8.02 (3H, m). ^{13}C-NMR ($CDCl_3$) δ: 154.5, 150.8, 144.0 (CH), 143.8 (CH), 142.6, 141.9, 141.3, 140.7, 137.1, 129.2 (CH), 125.1 (CH), 114.9 (CH), 112.9 (CH), 112.2 (CH), 111.9 (CH), 111.8 (CH), 45.3 (CH_2×2), 25.6 (CH_2×2), 24.2 (CH_2). IR (KBr) cm^{-1}: 3396, 3114, 2935, 2854, 1645, 1568, 1525, 1493, 1473, 1431, 1329, 1254, 1230. MS *m*/*z*: 388 (M^+), 303. EI-MS *m*/*z*: 388.1523 (Calcd for $C_{22}H_{20}N_4O_3$: 388.1535).

Compound **15**: A yellow amorphous solid, yield 86%. ^{1}H-NMR ($CDCl_3$) δ: 1.80 (4H, b), 3.56 (2H, t, J=4.9 Hz), 3.77 (2H, t, J=4.9 Hz), 6.56 (2H, m), 6.63 (1H, dd, J=0.8, 3.5 Hz), 6.66 (1H, dd, J=0.8, 3.5 Hz), 6.99 (1H, s), 7.60 (2H, m), 7.96—8.06 (3H, m). IR (KBr) cm^{-1}: 3423, 2920, 2852, 1653, 1529, 1475, 1429, 1333, 1254. MS *m*/*z*: 390 (M^+), 303. EI-MS *m*/*z*: 390.1309 (Calcd for $C_{21}H_{18}N_4O_4$: 390.1328).

Compound **16**: A brown amorphous solid, yield 95%. ^{1}H-NMR (CD_3OD) δ: 2.61 (3H, s), 2.89 (4H, t, J=5.0 Hz), 3.75 (4H, t, J=4.9 Hz), 6.60—6.67 (4H, m), 7.69 (2H, m), 7.88 (1H, dd, J=2.3, 9.1 Hz), 7.95 (1H, dd, J=0.3, 9.2 Hz), 8.17 (1H, d, J=2.4 Hz). IR (KBr) cm^{-1}: 3423, 2929, 2574, 1651, 1574, 1531, 1398, 1384, 1257, 1205. MS *m*/*z*: 403 (M^+), 303. EI-MS *m*/*z*: 403.1654 (Calcd for $C_{22}H_{21}N_5O_3$: 403.1644).

Compound **17**: A yellow amorphous solid, yield 54%. ^{1}H-NMR (DMSO-d_6) δ: 1.95 (1H, m), 3.26 (4H, d, J=6.0 Hz), 3.63 (2H, t, J=5.6 Hz), 3.76 (2H, s), 6.57—6.66 (4H, m), 7.76 (1H, dt, J=9.4, 1.9 Hz), 8.22 (1H, t, J=2.7 Hz), 8.88 (1H, s). IR (KBr) cm^{-1}: 3425, 2924, 1653, 1570, 1529, 1491, 1429, 1338, 1256, 1203. MS *m*/*z*: 403 (M^+), 303. EI-MS *m*/*z*:

403.1617 (Calcd for $C_{22}H_{21}N_5O_3$: 403.1644).

Compound **18**: A brown amorphous solid, yield 78%. ^{1}H-NMR ($CDCl_3$) δ: 1.11 (3H, t, J=7.1 Hz), 1.22 (3H, t, J=7.1 Hz), 1.50—2.00 (4H, m), 2.73 (1H, m), 3.30—3.46 (6H, m), 3.75 (1H, dt, J=13.2, 4.0 Hz), 3.95 (1H, dd, J=3.3, 13.7 Hz), 6.53 (2H, m), 6.58 (1H, d, J=3.4 Hz), 6.60 (1H, d, J=3.4 Hz), 7.58 (2H, m), 7.90—8.02 (4H, m). IR (KBr) cm^{-1}: 3425, 2933, 1618, 1529, 1475, 1431, 1256, 1232. MS m/z: 487 (M^+), 303. EI-MS m/z: 487.2222 (Calcd for $C_{27}H_{29}N_5O_4$: 487.2219).

Compound **19**: A brown amorphous solid, yield 44%. ^{1}H-NMR ($CDCl_3$) δ: 1.11 (3H, t, J=7.1 Hz), 1.22 (3H, t, J=7.1 Hz), 1.50—2.00 (4H, m), 2.73 (1H, m), 3.30—3.46 (6H, m), 3.75 (1H, dt, J=13.2, 4.0 Hz), 3.95 (1H, dd, J=3.3 Hz), 6.53 (2H, m), 6.58 (1H, d, J=3.4 Hz), 6.60 (1H, d, J=3.4 Hz), 7.58 (2H, m), 7.90—8.02 (4H, m). IR (KBr) cm^{-1}: 3423, 3114, 2972, 2933, 2870, 1616, 1570, 1529, 1493, 1431, 1385, 1331, 1306, 1256, 1232, 1205. MS m/z: 487 (M^+), 303. EI-MS m/z: 487.2219 (Calcd for $C_{27}H_{29}N_5O_4$: 487.2219).

Compound **20**: A brown amorphous solid, yield 36%. ^{1}H-NMR ($CDCl_3$) δ: 1.10 (3H, t, J=7.2 Hz), 1.28 (3H, t, J=7.2 Hz), 1.50—2.00 (6H, m), 3.16 (1H, m), 3.38 (2H, m), 3.54 (1H, m), 3.65 (1H, m), 4.94 (1H, td, J=11.8, 3.6 Hz), 5.27 (1H, m), 6.57 (1H, d, J=3.4 Hz), 6.61 (1H, d, J=3.6 Hz), 7.23 (1H, b), 7.58 (2H, m), 7.86 (1H, dd, J=2.4, 9.1 Hz), 7.99 (1H, d, J=9.1 Hz), 8.05 (1H, d, J=4.3 Hz). IR (KBr) cm^{-1}: 3386, 3342, 2937, 1657, 1622, 1568, 1531, 1495, 1385, 1338, 1256. MS m/z: 487 (M^+), 303, 84. EI-MS m/z: 487.2230 (Calcd for $C_{27}H_{29}N_5O_4$: 487.2220).

Compound **21**: A brown amorphous solid, yield 21%. ^{1}H-NMR ($CDCl_3$) δ: 1.08 (3H, t, J=7.2 Hz), 1.18 (3H, t, J=7.2 Hz), 1.70—1.90 (4H, m), 2.63 (1H, m), 2.97 (2H, t, J=12.2 Hz), 3.43 (4H, m), 4.19 (2H, d, J=13.5 Hz), 6.52 (2H, m), 6.56 (1H, dd, J=0.8, 3.4 Hz), 6.60 (1H, dd, J=0.8, 3.4 Hz), 7.40 (1H, b), 7.56 (2H, m), 7.90—8.00 (3H, m). IR (KBr) cm^{-1}: 3425, 2968, 2931, 2870, 1618, 1570, 1529, 1493, 1431, 1383, 1323, 1259, 1223. MS m/z: 487 (M^+), 303. EI-MS m/z: 487.2225 (Calcd for $C_{27}H_{29}N_5O_4$: 487.2219).

Compound **22**: A brown amorphous solid, yield 64%. ^{1}H-NMR ($CDCl_3$) δ: 1.29 (3H, t, J=6.1 Hz), 1.45—1.95 (3H, m), 2.20 (1H, m), 2.70 (1H, m), 3.10 (1H, m), 3.48 (1H, dd, J=3.3, 14.2 Hz), 3.99 (2H, m), 4.22 (2H, m), 6.53 (2H, m), 6.59 (2H, m), 7.59 (2H, m), 7.95 (2H, m), 8.02 (1H, m), 8.13 (1H, s). IR (KBr) cm^{-1}: 3386, 3116, 2937, 2860, 1728, 1649, 1570, 1529, 1493, 1475, 1431, 1331, 1256, 1230, 1203. MS m/z: 460 (M^+), 303. EI-MS m/z: 460.1717 (Calcd for $C_{25}H_{24}N_4O_5$: 460.1746).

Compound **23**: A brown amorphous solid, yield 64%. ^{1}H-NMR ($CDCl_3$) δ: 1.29 (3H, t, J=6.1 Hz), 1.45—1.95 (3H, m), 2.20 (1H, m), 2.70 (1H, m), 3.10 (1H, m), 3.48 (1H, dd, J=3.3, 14.2 Hz), 3.99 (2H, m), 4.22 (2H, m), 6.53 (2H, m), 6.59 (2H, m), 7.59 (2H, m), 7.95 (2H, m), 8.02 (1H, m), 8.13 (1H, s). IR (KBr) cm^{-1}: 3404, 2937, 2860, 1728, 1649, 1570, 1527, 1473, 1431, 1256. MS m/z: 460 (M^+), 303. EI-MS m/z: 460.1725 (Calcd for $C_{25}H_{24}N_4O_5$: 460.1747).

Compound **24**: A brown amorphous solid, yield 66%. ^{1}H-NMR ($CDCl_3$) δ: 1.29 (3H, t, J=6.1 Hz), 1.45—1.95 (3H, m), 2.20 (1H, m), 2.70 (1H, m), 3.10 (1H, m), 3.48 (1H, dd, J=3.3, 14.2 Hz), 3.99 (2H, m), 4.22 (2H, m), 6.53 (2H, m), 6.59 (2H, m), 7.59 (2H, m), 7.95 (2H, m), 8.02 (1H, m), 8.13 (1H, s). IR (KBr) cm^{-1}: 3404, 2937, 2860, 1728, 1649, 1570, 1529, 1475, 1431, 1254. MS m/z: 460 (M^+), 303. EI-MS m/z: 460.1738 (Calcd for $C_{25}H_{24}N_4O_5$: 460.1747).

Compound **25**: A yellow amorphous solid, yield 64%. ^{1}H-NMR ($CDCl_3$) δ: 1.13 (3H, t, J=7.1 Hz), 1.31 (3H, t, J=7.2 Hz), 1.90—2.35 (4H, m), 3.08—3.64 (5H, m), 3.79 (1H, m), 4.86 (1H, m), 6.52 (2H, m), 6.56 (1H, dd, J=0.8, 3.4 Hz), 6.60 (1H, dd, J=0.8, 3.4 Hz), 6.97 (1H, s), 7.57 (2H, m), 7.97 (2H, m), 8.00 (1H, m). IR (KBr) cm^{-1}: 3433, 3114, 2974, 2933, 2874, 1637, 1566, 1527, 1493, 1427, 1383, 1257, 1203. MS m/z: 473 (M^+), 303. EI-MS m/z: 473.2067 (Calcd for $C_{26}H_{27}N_5O_4$: 473.2063).

Compound **26**: A yellow amorphous solid, yield 98%. ^{1}H-NMR ($CDCl_3$) δ: 1.13 (3H, t, J=7.1 Hz), 1.31 (3H, t, J=7.2 Hz), 1.90—2.35 (4H, m), 3.08—3.64 (5H, m), 3.79 (1H, m), 4.86 (1H, m), 6.52 (2H, m), 6.56 (1H, dd, J=0.8, 3.4 Hz), 6.60 (1H, dd, J=0.8, 3.4 Hz), 6.97 (1H, s), 7.57 (2H, m), 7.97 (2H, m), 8.00 (1H, m). IR (KBr) cm^{-1}: 3438, 3311, 3113, 2974, 2933, 2874, 1668, 1637, 1566, 1529, 1429, 1383, 1257, 1203. MS m/z: 473 (M^+), 303. EI-MS m/z: 473.2052 (Calcd for $C_{26}H_{27}N_5O_4$: 473.2063).

Compound **27**: A brown amorphous solid, yield 70%. ^{1}H-NMR ($CDCl_3$) δ: 1.04 (3H, t, J=7.0 Hz), 1.34 (3H, t, J=7.0 Hz), 3.02—3.24 (2H, m), 3.48 (4H, m), 4.79 (1H, d, J=14.0 Hz), 4.95 (1H, d, J=14.0 Hz), 5.37 (1H, t, J=5.8 Hz), 6.53 (2H, m), 6.58 (1H, dd, J=0.7, 3.4 Hz), 6.62 (1H, dd, J=0.8, 3.6 Hz), 7.14—7.22 (4H, m), 7.41 (1H, s), 7.58 (2H, m), 8.00 (1H, d, J=9.1 Hz), 8.07 (1H, d, J=2.3 Hz). IR (KBr) cm^{-1}: 3425, 2972, 2931, 1635, 1568, 1529, 1495, 1427, 1327, 1242. ESI-MS m/z: 536.2272 (Calcd for $C_{31}H_{30}N_5O_4$: 536.2298).

Compound **28**: A yellow amorphous solid, yield 66%. ^{1}H-NMR ($CDCl_3$) δ: 1.09 (9H, s), 3.24 (2H, b), 4.50—4.86 (3H, m), 5.46 (1H, b), 6.50—6.70 (4H, m), 7.20—7.40 (4H, b), 7.60 (2H, b), 7.92 (1H, b), 8.05 (2H, b), 8.17 (1H, b). ^{13}C-NMR ($CDCl_3$) δ: 171.0, 155.5, 150.9, 144.1 (CH), 143.9 (CH), 142.9, 141.5, 141.0, 137.4, 133.8, 133.2, 129.5 (CH), 128.1 (CH), 127.9 (CH), 127.5 (CH), 126.7 (CH), 124.8 (CH), 115.8 (CH), 112.9 (CH), 112.3 (CH), 111.9 (CH), 111.8 (CH), 57.0 (CH), 51.4, 44.9 (CH_2), 31.9 (CH_2), 28.3 (CH_3×3). IR (KBr) cm^{-1}: 3415, 3336, 2968, 1647, 1570, 1531, 1496, 1385, 1363, 1327, 1256. MS m/z: 535 (M^+), 303, 132. EI-MS m/z: 535.2210 (Calcd for $C_{31}H_{29}N_5O_4$: 535.2219).

Compound **29**: A yellow amorphous solid, yield 59%. ^{1}H-NMR ($CDCl_3$) δ: 1.11 (3H, t, J=7.1 Hz), 1.21 (3H, t, J=7.1 Hz), 3.33 (2H, q, J=7.0 Hz), 3.40 (2H, q, J=7.0 Hz), 4.22 (2H, d, J=4.6 Hz), 6.40—6.58 (4H, m), 6.87 (1H, t, J=4.5 Hz), 7.52 (2H, s), 7.79 (2H, m), 8.02 (1H, d, J=2.0 Hz), 8.77 (1H, d, J=4.7 Hz). IR (KBr) cm^{-1}: 3350, 3113, 2974, 2933, 1701, 1624, 1570, 1539, 1491, 1437, 1336, 1221. MS m/z: 433 (M^+), 303. EI-MS m/z: 433.1742 (Calcd for $C_{23}H_{23}N_5O_4$: 433.1750).

Compound **30**: A yellow amorphous solid, yield 69%. ^{1}H-NMR ($CDCl_3$) δ: 1.08 (3H, t, J=7.1 Hz), 1.19 (3H, t, J=7.1 Hz), 2.70 (2H, t, J=5 Hz), 3.35 (4H, m), 3.65 (2H, m), 6.40—6.58 (5H, m), 7.58 (2H, s), 7.93 (1H, b), 7.99 (1H, d, J=9.2 Hz), 8.14 (1H, dd, J=2.2, 9.2 Hz), 8.76 (1H, b). ^{13}C-NMR ($CDCl_3$) δ: 171.8, 155.6, 151.0, 150.9, 144.0 (CH), 143.7 (CH), 142.8, 142.5, 141.7, 140.4, 136.9, 129.5 (CH), 124.1 (CH), 113.5 (CH), 112.8 (CH), 112.0 (CH), 111.8 (CH), 111.8 (CH), 42.2 (CH_2), 40.7 (CH_2), 35.7 (CH_2), 33.4 (CH_2), 14.0 (CH_3), 13.2 (CH_3). IR (KBr) cm^{-1}: 3406, 3298, 3124, 2982, 1711, 1606, 1576, 1539, 1522, 1493, 1468, 1383, 1336, 1202. MS m/z: 447 (M^+), 303. EI-MS m/z: 447.1910 (Calcd for $C_{24}H_{25}N_5O_4$: 447.1907).

Compound **31**: A brown amorphous solid, yield 63%. ^{1}H-NMR ($CDCl_3$) δ: 1.09 (3H, t, J=7.1 Hz), 1.15 (3H, t, J=7.1 Hz), 1.46—1.67 (6H, m), 2.30 (2H, t, J=7.3 Hz), 3.22—3.40 (6H, m), 6.26 (1H, t, J=5.5 Hz), 6.48—6.57 (4H, m), 7.56 (2H, d, J=4.3 Hz), 7.98 (3H, m), 8.59 (1H, b). IR (KBr) cm^{-1}: 3350, 3138, 2931, 2862, 1701, 1614, 1541, 1493, 1431, 1336, 1225. MS m/z: 489 (M^+), 303. EI-MS m/z: 489.2366 (Calcd for $C_{27}H_{31}N_5O_4$: 489.2376).

Compound **32**: A brown amorphous solid, yield 66%. ^{1}H-NMR ($CDCl_3$) δ: 1.22 (3H, t, J=7.2 Hz), 4.08 (2H, d, J=5.6 Hz), 4.15 (2H, q, J=7.2 Hz), 6.23 (1H, t, J=5.5 Hz), 6.44—6.54 (4H, m), 7.52 (2H, m), 7.84 (2H, m), 7.93 (1H, s), 8.42 (1H, s). IR (KBr) cm^{-1}: 3381, 3316, 2980, 2931, 1740, 1689, 1672, 1618, 1572, 1543, 1495, 1473, 1408, 1377, 1338, 1306, 1259, 1207. MS m/z: 406 (M^+), 303. EI-MS m/z: 406.1242 (Calcd for $C_{21}H_{18}N_4O_5$: 406.1278).

Compound **33**: A brown amorphous solid, yield 64%. ^{1}H-NMR ($CDCl_3$) δ: 1.08 (3H, t, J=7.2 Hz), 1.14 (3H, t, J=7.1 Hz), 3.10—3.30 (5H, m), 3.68 (1H, m), 5.20 (1H, m), 6.51 (3H, m), 8.48 (1H, s), 6.61 (1H, dd, J=0.7, 3.4 Hz), 7.26 (5H, m), 7.44 (1H, d, J=8.8 Hz), 7.56 (1H, s), 7.78 (1H, dd, J=2.3, 9.1 Hz), 7.91 (1H, d, J=9.1 Hz), 8.08 (1H, d, J=2.2 Hz). IR (KBr) cm^{-1}: 3346, 2974, 2933, 1703, 1618, 1537, 1493, 1336, 1225. MS m/z: 523 (M^+), 303, 120. EI-MS m/z: 523.2209 (Calcd for $C_{30}H_{29}N_5O_4$: 523.2219).

Compound **34**: A brown amorphous solid, yield 55%. ^{1}H-NMR ($CDCl_3$) δ: 1.08 (3H, t, J=7.2 Hz), 1.14 (3H, t, J=7.1 Hz), 3.10—3.30 (5H, m), 3.68 (1H, m), 5.20 (1H, m), 6.51 (3H, m), 6.61 (1H, dd, J=0.7, 3.4 Hz), 7.26 (5H, m), 7.44 (1H, d, J=8.8 Hz), 7.56 (1H, s), 7.78 (1H, dd, J=2.3, 9.1 Hz), 7.91 (1H, d, J=9.1 Hz), 8.08 (1H, d, J=2.2 Hz), 8.48 (1H, s). IR (KBr) cm^{-1}: 3346, 2974, 2931, 1703, 1618, 1568, 1539, 1493, 1336, 1226. MS m/z: 523 (M^+), 303, 120. EI-MS m/z: 523.2256 (Calcd for $C_{30}H_{29}N_5O_4$: 523.2219).

Compound **35**: A brown amorphous solid, yield 65%. ^{1}H-NMR ($CDCl_3$) δ: 0.80—1.40 (14H, m), 1.65—1.90 (1H, m), 3.30—3.75 (4H, m), 4.79 (1H, t, J=9.3 Hz), 6.52 (3H, b), 6.62 (1H, d, J=3.2 Hz), 7.12 (1H, d, J=9.6 Hz), 7.56 (2H, s), 7.88 (1H, d, J=9.1 Hz), 7.96 (1H, d, J=9.2 Hz), 8.10 (1H, d, J=1.8 Hz), 8.62 (1H, s). IR (KBr) cm^{-1}: 3348, 2968, 2933, 2875, 1703, 1612, 1568, 1539, 1493, 1466, 1383, 1336, 1308, 1259, 1209. MS m/z: 489 (M^+), 303. EI-MS m/z: 489.2395 (Calcd for $C_{27}H_{31}N_5O_4$: 489.2376).

Compound **36**: A brown amorphous solid, yield 62%. ^{1}H-NMR ($CDCl_3$) δ: 0.84 (3H, t, J=7.3 Hz), 0.91 (3H, d, J=6.7 Hz), 1.00—1.45 (2H, m), 2.26 (1H, b), 3.73 (3H, s), 4.56 (1H, dd, J=5.1, 8.5 Hz), 6.05 (1H, d, J=8.2 Hz), 6.46—6.57 (4H, m), 7.56 (2H, dd, J=1.0, 8.8 Hz), 7.90 (3H, m), 8.18 (1H, s). IR (KBr) cm^{-1}: 3381, 2964, 2933, 2877, 1740, 1664, 1618, 1568, 1541, 1495, 1435, 1385, 1336, 1261, 1209. MS m/z: 448 (M^+), 416, 303. EI-MS m/z: 448.1750 (Calcd for $C_{24}H_{24}N_4O_5$: 448.1747).

Compound **37**: A brown amorphous solid, yield 65%. ^{1}H-NMR ($CDCl_3$) δ: 1.21 (3H, t, J=7.1 Hz), 1.40 (3H, t, J=7.1 Hz), 1.98 (2H, m), 2.14 (3H, s), 2.66 (2H, m), 3.39 (2H, q, J=7.0 Hz), 3.59 (2H, q, J=7.4 Hz), 5.12 (1H,

m), 6.49 (3H, m), 6.58 (1H, dd, J=0.7, 3.3 Hz), 7.21 (1H, b), 7.54 (2H, m), 7.74 (1H, dd, J=2.3, 9.2 Hz), 7.86 (1H, d, J=9.2 Hz), 8.03 (1H, d, J=2.3 Hz), 8.31 (1H, d, J=6.0 Hz). IR (KBr) cm^{-1}: 3352, 3114, 2974, 2931, 1701, 1618, 1568, 1539, 1493, 1437, 1385, 1336, 1308, 1259, 1225. MS *m*/*z*: 507 (M^+), 303. EI-MS *m*/*z*: 507.1957 (Calcd for $C_{26}H_{29}N_5O_4S$: 507.1940).

Compound **38**: A yellow amorphous solid, yield 99%. ^{1}H-NMR ($CDCl_3$) δ: 1.13 (6H, m), 1.71 (2H, m), 1.90 (2H, m), 2.74 (1H, m), 3.15 (2H, m), 3.38 (4H, m), 4.21 (1H, d, J=4.0 Hz), 4.33 (1H, d, J=4.1 Hz), 6.57 (2H, m), 6.68 (2H, m), 7.60 (2H, m), 7.71 (2H, m), 8.50 (1H, m), 9.16 (1H, s). IR (KBr) cm^{-1}: 3379, 3107, 2972, 2933, 2858, 1672, 1622, 1570, 1537, 1487, 1468, 1446, 1404, 1383, 1336, 1256, 1225. MS *m*/*z*: 487 (M^+), 303. EI-MS *m*/*z*: 487.2209 (Calcd for $C_{27}H_{29}N_5O_4$: 487.2219).

Compound **39**: A yellow amorphous solid, yield 81%. ^{1}H-NMR ($CDCl_3$) δ: 1.10 (3H, t, J=7.1 Hz), 1.22 (3H, t, J=7.2 Hz), 1.80—2.03 (4H, m), 2.70 (1H, m), 3.11 (2H, td, J=12.6, 3.0 Hz), 3.37 (4H, m), 4.29 (2H, d, J=10.3 Hz), 6.55 (2H, m), 6.66 (2H, m), 7.57 (1H, m), 7.61 (1H, m), 7.70 (2H, m), 8.48 (1H, m), 9.09 (1H, s). IR (KBr) cm^{-1}: 3392, 3109, 2972, 2931, 2860, 1668, 1630, 1570, 1533, 1489, 1448, 1404, 1383, 1336, 1265, 1219. MS *m*/*z*: 487 (M^+), 303. EI-MS *m*/*z*: 487.2235 (Calcd for $C_{27}H_{29}N_5O_4$: 487.2220).

Compound **40**: A yellow amorphous solid, yield 69%. ^{1}H-NMR ($CDCl_3$) δ: 0.98 (6H, d, J=6.6 Hz), 1.65 (2H, m), 1.78 (1H, m), 4.29 (2H, t, J=6.9 Hz), 6.56 (1H, m),8.81 (1H, s), 6.61 (2H, m), 6.78 (1H, m), 7.63 (2H, b), 7.76 (2H, m), 8.44 (1H, b). IR (KBr) cm^{-1}: 3369, 3116, 2958, 2870, 1736, 1645, 1614, 1572, 1525, 1491, 1470, 1444, 1402, 1385, 1338, 1309, 1279, 1203. MS *m*/*z*: 391 (M^+), 303. EI-MS *m*/*z*: 391.1525 (Calcd for $C_{22}H_{21}N_3O_4$: 391.1532).

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