Minor Structural Differences in Boc-CCK-4 Derivatives Dictate Affinity and Selectivity for CCK-A and CCK-B Receptors

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We previously reported novel Boc-CCK-4 (Boc-Trp-Met-Asp-Phe-NH_2) derivatives possessing the general structure Boc-Trp-Lys[N^{ϵ}-CO-NH-(R-Ph)]-Asp-Phe-NH_2 (Shiosaki et al. *J. Med. Chem.* **1991**, *34*, 2837−2842). In contrast to Boc-CCK-4, which is 70-fold selective for the CCK-B receptor, the modified lysine-bearing tetrapeptides were highly potent and selective full agonists at the CCK-A receptor. Further investigation of the structure−activity profile following modification of the substituted phenylurea moiety appended off the lysine revealed that moving certain substituents, e.g. nitro or acetyl, from the 2- or 3-position on the phenyl ring to the 4-position, a relatively minor and subtle structural modification within the tetrapeptide, resulted in loss of CCK-A receptor selectivity and development of a trend toward CCK-B selectivity. These tetrapeptides, e.g. Boc-Trp-Lys[N^{ϵ}-CO-NH-(4-NO_2-Ph)]-Asp-Phe-NH_2 and Boc-Trp-Lys[N^{ϵ}-CO-NH-(4-Ac-Ph)]-Asp-Phe-NH_2, were full agonists relative to CCK-8 in stimulating intracellular calcium mobilization in a cell line that expresses the CCK-B receptor.

Cholecystokinin (CCK) is a 33-amino acid peptide that is found in the central nervous system (CNS) as well as in peripheral tissues of various mammalian species, including humans.[1,2] In the CNS, high concentrations of CCK-8, the sulfated C-terminal octapeptide of CCK, have been identified in cerebral cortex, hippocampus, midbrain, and spinal neurons.[3] The coexistence and interaction of CCK with other neurotransmitters, e.g. dopamine and GABA,[3,4] and neuropeptides, e.g. neurotensin and substance P,[3] support an involvement of the peptide in regulating CNS function. Changes in the levels of CCK found in tissues and sera of patients with various disease states, including schizophrenia[5] and eating disorders,[6] and the effects of CCK derivatives in models of Parkinson's disease,[7] anxiety,[8] and pain[9] indicate the potential utility of CCK-based compounds as therapeutic agents. Additionally, CCK is distributed throughout the periphery where it is involved in the regulation of pancreatic and bile secretions, gallbladder contraction, gut motility, gastric emptying, and satiety.[10]

The biological actions of CCK are mediated by two receptor subtypes, specified as CCK-A and CCK-B. The CCK-A receptor subtype binds sulfated CCK-8 with significantly higher affinity than either the desulfated form of CCK-8 or CCK-4, whereas the CCK-B receptor binds all three of these CCK-related peptides with comparable affinities. In addition, a number of synthetic peptides and non-peptide ligands that are selective for either the CCK-A (MK-329 (devazepide), CR-1409, A-71378, A-71623)[11−13] or the CCK-B receptor (PD134308 and L-365,260)[14,15] have been developed.

Both the CCK-A and CCK-B receptors have been cloned from rat, guinea pig, and human tissues. Each receptor subtype is highly conserved (>90%) between species, with the greatest degree of homology observed in the transmembrane domains.[16] The high degree of homology is reflected in the similarity of their pharmacological profiles. The CCK-A receptor is the predominant subtype found in peripheral tissues such as the pancreas, colon, and gallbladder, although low levels of this subtype are found in discrete regions of the CNS.[17] The CCK-B receptor is the main subtype found in the CNS and appears to be the same gene product as the peripheral gastrin receptor.[18]

We reported on a series of tetrapeptides (**1**) that possess high affinity and selectivity for the CCK-A receptor.[13,19] These tetrapeptides are derivatives of Boc-CCK-4 (Boc-Trp-Met-Asp-Phe-NH_2), in which the methionine is replaced by a lysine residue whose N^{ϵ}-amino is incorporated into various substituted phenylureas. Whereas Boc-CCK-4 possesses high affinity (IC_{50} = 25 nM) and selectivity (70-fold) for the CCK-B receptor, the lysine-substituted tetrapeptide derivatives (e.g. R = H, Me, Cl, CF_3) possess high affinity and selectivity for the CCK-A receptor. In addition, these derivatives function as full agonists in stimulating pancreatic amylase release that are blocked using selective CCK-A antagonists.[20]

This report describes the further investigation of the structure−activity profile related to substitutions on the phenylurea moiety in **1**. We observed that by changing the placement of certain substituents at the 2-, 3-, or 4-position of the phenyl ring, relatively minor and subtle structural modifications within the entire structure of the tetrapeptide, a loss in CCK-A receptor selectivity and a trend toward CCK-B selectivity were achieved.

Methods

The tetrapeptide **4** was prepared by standard solution-phase peptide chemistry techniques. The side-chain amino group of lysine in **4** was reacted with an aryl isocyanate to yield the final peptides (Scheme 1). All compounds were purified by chromatography, and the spectral and elemental analyses for all final compounds were consistent with their structures.

The tetrapeptides (**1**) were tested in receptor binding assays using guinea pig pancreas and cortex as tissues containing the CCK-A and CCK-B receptors, respectively, and [I^{125}]Bolton−

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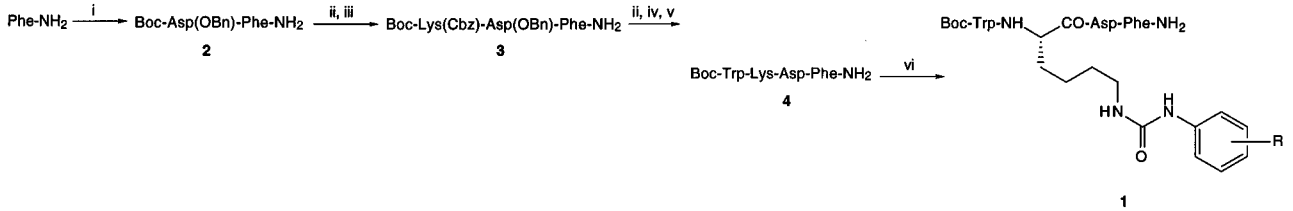
Scheme 1[a]



[a] Reagents: (i) Boc-Asp(OBn)-OH, EDCI, HOBt; (ii) HCl in HOAc; (iii) Boc-Lys(Cbz)-OH, EDCI, HOBt, *N*-methylmorpholine; (iv) Boc-Trp hydroxysuccinimide ester, *N*-methylmorpholine; (v) H_2, 10% Pd−C; (vi) R-Ph-NCO, *N*-methylmorpholine.

Table 1. Physical Properties and Binding Data of Lysine-Substituted Tetrapeptide **1**

					IC_{50}, nM[b,c]		
no.	R	mp, °C	formula	anal.[a]	pancreas	cortex	C/P[d]
1a	2-Me	172−174	$C_{43}H_{54}N_8O_9 \cdot AcOH$	C,H,N	3.8 ± 0.49 (3)	1400 ± 490 (3)	370
1b	3-Me	189−191	$C_{43}H_{54}N_8O_9 \cdot 0.75H_2O \cdot AcOH$	C,H,N	16 ± 3.1 (3)	1900 ± 240 (3)	120
1c	4-Me	144−145	$C_{43}H_{54}N_8O_9 \cdot 1.75H_2O \cdot AcOH$	C,H,N	53 ± 24 (4)	1200 ± 50 (3)	23
1d	2-MeO	140−142	$C_{43}H_{54}N_8O_{10} \cdot 0.5H_2O$	C,H,N	23 ± 5.3 (4)	3400 ± 500 (4)	150
1e	3-MeO	132−133	$C_{43}H_{54}N_8O_{10} \cdot H_2O \cdot 0.5AcOH$	C,H,N	9.4 ± 1.2 (3)	1400 ± 130 (3)	150
1f	4-MeO	153−155	$C_{43}H_{54}N_8O_{10} \cdot 2H_2O$	C,H,N	14 ± 6.8 (3)	750 ± 140 (3)	50
1g	2-CO_2Me	191−192	$C_{44}H_{54}N_8O_{11} \cdot 0.5H_2O \cdot 0.5AcOH$	C,H,N	34 ± 4.5 (3)	3100 ± 590 (3)	91
1h	3-CO_2Me	200−202	$C_{44}H_{54}N_8O_{11} \cdot 1.75H_2O$	C,H,N	15 ± 7.6 (4)	1000 ± 180 (3)	67
1i	4-CO_2Me	136−138	$C_{44}H_{54}N_8O_{11} \cdot 1.75H_2O$	C,H,N	71 ± 46 (3)	150 ± 100 (3)	2.1
1j	2-NO_2	123−124	$C_{42}H_{51}N_9O_{11} \cdot H_2O$	C,H,N	22 ± 3.9 (4)	820 ± 61 (4)	37
1k	3-NO_2	140−142	$C_{42}H_{51}N_9O_{11} \cdot 2H_2O$	C,H,N	5.8 ± 1.7 (3)	1200 ± 290 (3)	210
1l	4-NO_2	nd[e]	$C_{42}H_{51}N_9O_{10} \cdot 1.5H_2O \cdot AcOH$	C,H,N	41 ± 5.6 (4)	31 ± 4.3 (9)	0.75
1m	3-Ac	135−137	$C_{44}H_{54}N_8O_{10} \cdot H_2O$	C,H,N	19 ± 4.5 (3)	1100 ± 91 (3)	250
1n	4-Ac	187−189	$C_{44}H_{54}N_8O_{10} \cdot 1.25H_2O$	C,H,N	46 ± 17 (9)	12 ± 3.1 (3)	0.26

[a] Compounds gave satisfactory analysis within ±0.4% of theoretical calculations. [b] IC_{50} was determined as the concentration of peptide that inhibited 50% of the specific binding of [^{125}I]BH-CCK-8 in each tissue. [c] IC_{50} values represent means ± SE. Number of determinations is indicated in parentheses and each determination was conducted in duplicate with <5% sample variability. [d] IC_{50} cortex/IC_{50} pancreas. [e] Not determined.

Hunter CCK-8 as the radioligand as previously described.[12] The IC_{50} value was determined as the concentration of the peptide that inhibited 50% of the specific binding of [I^{125}]Bolton−Hunter CCK-8 in each tissue. Protocols for assessing the ability of these compounds to mobilize intracellular calcium levels in NCI-H345 cells, which contains the CCK-B/gastrin receptor, using the fluorescent probe indo-1 has been described previously.[12] Protocols for assessing the ability of these compounds to stimulate amylase release and phosphatidyl inositide (PI) hydrolysis in guinea pig pancreas have been described.[12]

Results and Discussion

We previously reported on tetrapeptides derivatives of **1** in which the phenylurea was monosubstituted at the 2-, 3-, or 4-position with a methyl, chloro, or trifluoromethyl group.[19] These derivatives all possessed selectivity for the CCK-A (pancreatic) over the CCK-B (cortical) receptors. We also noted that with a given substituent, the 2-substitutions generated compounds with the highest affinity and greatest degree of selectivity (200−500-fold) for the CCK-A receptor, with selectivity progressively decreasing as the substituent is moved from the 2- to the 3- and then to the 4-position. Substitutions at the 4-position produced derivatives that were 5−20-fold CCK-A selective. This trend was primarily a result of decreased CCK-A affinity, although minor concomitant increase in CCK-B affinity was observed in some examples upon moving the substituent stepwise from the 2- to the 4-position of the phenyl ring.

On the basis of the above observations, the structure−activity studies of substituents on the phenylurea in **1** were expanded to include additional functional groups (Table 1). The binding data for the previously described methyl derivatives **1a**−**c** are included for reference.[19] The methoxy isomers **1d**−**f** possessed high affinity and selectivity for the CCK-A receptor. In contrast to the methyl series (**1a**−**c**), in which a greater than 10-fold decrease in CCK-A receptor affinity was observed in moving the substituent from the 2- to the 4-position, no difference in CCK-A receptor binding was noted among the three positional methoxy isomers. In addition, CCK-B receptor binding was not affected by the position of the methyl group in **1**, whereas a nearly 5-fold increase in CCK-B affinity was observed by moving the methoxy group from the 2- to the 4-position.

The methoxycarbonyl isomers (**1g**−**i**) produced a trend in binding affinities somewhat similar to that observed with the methoxy derivatives (**1d**−**f**). However, the 4-methoxycarbonyl derivative (**1i**) no longer exhibited a significant binding preference for the CCK-A receptor in contrast to the 50-fold CCK-A selectivity of the 4-methoxy derivative (**1f**).

The introduction of a nitro or acetyl group at the 4-position of the phenylurea in **1** yielded tetrapeptides **1l** and **1n,** respectively, that possessed significantly greater affinity (IC_{50} = 31 and 12 nM, respectively) for the CCK-B receptor than for any of the other substituted phenylurea derivatives tested to date.[13,19] The increase in CCK-B affinity coupled with the decrease in CCK-A binding resulted in these tetrapeptides exhibiting a trend toward preferential CCK-B receptor binding. The 4-acetyl derivative **1n** appears to have the greatest binding selectivity for the CCK-B receptor of all the derivatives prepared. However, there is no statistically significant difference between the binding affinity (IC_{50}) of **1n** for the pancreatic CCK-A versus the cortical CCK-B receptors ($P > 0.1$, unpaired *t*-test). Comparison of binding data associated with the 4-acetyl (**1n**) and

Table 2. Ability of Tetrapeptides to Mobilize Intracellular Calcium Relative to CCK-8 in the NCI-H345 Cell

no.	EC_{50} (nM)[a]	% maximal response[b]
1l	47 ± 6.3 (6)	94 ± 2.2 (6)
1n	120 ± 12 (6)	91 ± 7.1 (6)

[a] EC_{50} was determined as the concentration of the peptide that produced 50% of the maximal response in intracellular calcium mobilization. The EC_{50} values represent means ± SE, and the number of determinations is indicated in parentheses. [b] Indicates the maximal response elicited by the peptides relative to the maximal response elicited by CCK-8. The number of determinations is indicated in parentheses.

the 4-methyl (**1c**) derivatives revealed that both compounds possessed similar binding affinities for the CCK-A receptor, whereas **1n** had 100-fold greater affinity for the CCK-B receptor than **1c**.

On the basis of the unexpectedly high CCK-B receptor affinity exhibited by **1l** and **1n**, both compounds were tested in a functional assay using a human small-cell lung carcinoma cell line (NCI-H345) that expresses the CCK-B/gastrin receptor. CCK-8 stimulated intracellular calcium mobilization in the H345 cells as measured by indo-1 fluorescence with an EC_{50} of 3.5 nM.[12] This response was blocked by a CCK-B selective antagonist (L-365,260).[12] Compounds **1l** and **1n** behaved as full agonists relative to CCK-8 in mobilizing internal calcium in the H345 cell assay (Table 2). Maximal response by **1l** (94% of CCK-8) was observed at a drug concentration of 1 μM, whereas the maximal response by **1n** (91%) was elicited at 10 μM. Although the 4-acetyl derivative (**1n**) had higher affinity than the 4-nitro compound (**1l**) for the CCK-B receptor, **1n** was less potent than **1l** in the calcium mobilization assay. A concentration of 100 nM of L-365,260, a CCK-B selective antagonist, inhibited 66% and 88% of the calcium response induced by 1 μM **1l** and **1n**, respectively, whereas MK-329 (100 nM), a CCK-A selective antagonist, did not inhibit any of the response induced by **1l** (1 μM) and inhibited 15% of the calcium response elicited by **1n** (1 μM).

In addition, **1l** and **1n** were assessed in functional assays (amylase release and phosphatidyl inositide (PI) hydrolysis) that are known to be mediated via CCK-A receptor stimulation.[21] Both **1l** and **1n** functioned as full agonists relative to CCK-8 in stimulating amylase release in guinea pig pancreatic acini. The EC_{50} values for **1l** and **1n** were 39 and 85 nM, respectively. In the PI hydrolysis assay, a concentration of 100 μM of **1l** stimulated 74% of the CCK-8 (1 μM) response, whereas **1n** (100 μM) stimulated 80%.

Benzodiazepine-[15,22] and dipeptoid-[23] based CCK ligands can be modified to yield derivatives that prefer either the CCK-A or CCK-B receptor. In many of these series, inversion of chiral centers were typically required in order to significantly affect receptor selectivity. In the case of the tetrapeptides **1**, changing the placement of certain substituents at the 2-, 3-, or 4-position of the phenyl ring, relatively minor and subtle structural modifications within the entire structure of the tetrapeptide, were sufficient to significantly affect receptor subtype affinity. Detailed molecular modeling studies are currently being undertaken to better understand how the various classes of CCK ligands are interacting with the receptor subtypes to dictate selectivity.

Experimental Section

Solvents and other reagents were reagent grade and used without further purification unless otherwise noted. Amino acids and *tert*-Butoxycarbonyl (Boc) protected amino acids were purchased from Bachem, Inc., Torrance, CA, or Sigma Chemical Co., St. Louis, MO. The activated esters (*N*-hydroxysuccinimide) of Boc-protected amino acids were purchased from Chemical Dynamics, South Plainfield, NJ. Melting points are uncorrected and were obtained on a Buchi capillary melting point apparatus. ^{1}H-NMR spectra were recorded at 300 or 500 MHz and expressed as ppm downfield from tetramethylsilane (TMS) as an internal standard. Column chromatography was performed on silica gel 60, 0.04−0.063 mm (E. Merck), using the following solvent system: ethyl acetate−pyridine−acetic acid−water (260:20:6:11). Pyridine used as the chromatography solvent was freshly distilled from barium oxide. Elemental analyses were performed by the Abbott Laboratories Analytical Department, North Chicago, IL, and are within ±0.4% of calculated values unless otherwise noted. The following abbreviations have been used: Z, benzyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; EDCI, 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride; HOBt, 1-hydroxybenzotriazole. Other peptide and amino acid abbreviations and conventions used are those recommended by the IUPAC−IUB Commission (*Biochem. J.* **1984**, *219*, 345−373).

Boc-Asp(OBzl)-Phe-NH_2 (2).[24] To a solution of phenylalaninamide hydrochloride (19.4 g, 0.06 mol) in dimethylformamide (100 mL) cooled to 0 °C were added *N*-methylmorpholine (7.2 mL, 0.065 mol), a solution of Boc-Asp β-benzyl ester (12.0 g, 0.06 mol) in methylene chloride (80 mL), HOBt (12.2 g, 0.09 mol), and EDCI (12.4 g, 0.065 mol). The reaction mixture was stirred for 12 h with warming to ambient temperature. The solvent was removed in vacuo and the resulting residue was dissolved in ethyl acetate and washed with 1 M H_3PO_4 (3×), saturated sodium bicarbonate ($NaHCO_3$) solution (3×), and brine. After drying ($MgSO_4$), the solvent was evaporated, the residue was dissolved in hot ethyl acetate, and the product precipitated with dropwise addition of hexane. The product was collected and dried to yield **2** (25 g, 88%) as a white solid: MS(CI/NH_3) *m/e* 470 $(M + H)^+$, 487; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.39 (s, 9H), 2.78 (dd, J = 18 Hz, 1H), 2.92−3.05 (m, 2H), 3.21 (dd, 1H), 4.38−4.45 (m, 1H), 4.65 (q, J = 6 Hz, 1H), 5.36 (br s, 1H), 5.49 (br d, J = 7.5 Hz, 1H), 6.09 (br s, 1H), 6.82 (br d, J = 7 Hz, 1H), 7.21−7.40 (m, 10H).

Boc-Lys(Cbz)-Asp(OBzl)-Phe-NH_2 (3). A solution of **2** (16.2 g, 34 mmol) in 50 mL of 1.5 M HCl (anhydrous) in acetic acid was stirred at ambient temperature for 1.5 h. The reaction was quenched with the addition of ether to precipitate the hydrochloride salt of the amine. The solid was collected, washed with fresh ether, and dried to yield 12.9 g of a white powder. To a solution of the hydrochloride salt (12.9 g, 32 mmol) in dimethylformamide (20 mL) and methylene chloride (20 mL) cooled to −10 °C were added *N*-methylmorpholine (3.9 mL, 35 mmol), Boc-Lys(Cbz) (12.1g, 32 mmol), HOBt (6.5 g, 48 mmol), and EDCI (6.7g, 35 mmol). The reaction was stirred for 12 h with warming to ambient temperature. The solvents were removed in vacuo, and the residue was dissolved in ethyl acetate and washed successively with solutions of 1 M H_3PO_4 (3×), saturated $NaHCO_3$ (3×), and brine. The solvent was removed in vacuo, and the solid residue was dissolved in acetone with warming. The product was precipitated with the dropwise addition of water, collected, and dried to yield **3** (22.3 g, 90%) as a white powder: MS(CI/NH_3) *m/e* 732 $(M + H)^+$, 749, 632; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 1.13−1.54 (m, 6H), 1.37 (br s, 9H), 2.51−3.05 (m, 6H), 3.86 (br s, 1H), 4.38 (br s, 1H), 4.61 (br s, 1H), 5.00 (s, 2H), 5.07 (s, 2H), 6.87 (br d, J = 7 Hz, 1H), 7.12−7.38 (m, 16H), 7.85 (br d, 1H), 8.15 (br d, 1H). Anal. Calcd for $C_{39}H_{49}N_4O_9$: C, 64.00; H, 6.75; N, 9.57. Found: C, 63.92; H, 6.82; N, 9.54.

Boc-Trp-Lys-Asp-Phe-NH_2 (4). A solution of **3** (19 g, 26 mmol) in 80 mL of 1.5 M HCl (anhydrous) in acetic acid was stirred at ambient temperature for 1.5 h. The product was precipitated with the addition of ether, collected, and dried to yield 17.2 g of a white powder. To a solution of the hydrochloride salt (9.8 g, 15.5 mmol) in dimethylformamide (100 mL)

cooled to 0 °C were added *N*-methylmorpholine (1.9 mL, 17 mmol) and Boc-tryptophan *N*-hydroxysuccinimide ester (6.15 g, 15.5 mmol). The reaction mixture was stirred for 18 h with warming to ambient temperature. The solvent was removed in vacuo and the residue partitioned between a solution of 10% citric acid and ethyl acetate. The organic phase was further washed with solutions of saturated $NaHCO_3$ (3×) and water (3×). After the mixture was dried over $MgSO_4$, the solvent was removed in vacuo. The solid residue was dissolved in ethyl acetate−acetone and the product precipitated with the addition of water. The product was collected and dried to yield 11.9 g of a white solid. A mixture of the fully protected tetrapeptide (5.0 g, 5.45 mmol) and 10% Pd/C (1.0 g) in acetic acid (100 mL) was hydrogenated for 18 h under 1 atm of hydrogen at ambient temperature. The catalyst was filtered and the solvent was removed in vacuo. The residue was triturated with ether to yield 3.95 g of **4** as a light pink powder: MS (FAB^+) *m/e* 694 $(M + H)^+$; ^1H NMR (DMSO-d_6, 300 MHz) δ 1.11−1.62 (m, 6H), 1.32 (br s, 1H), 2.25−3.18 (m, 8H), 4.10−4.43 (m, 4H), 6.78−7.34 (m, 12H), 7.51−7.62 (m, 2H), 8.05 (br d, $J = 7$ Hz, 1H), 8.17 (br d, $J = 7$ Hz, 1H), 10.91 (br s, 1H). Anal. Calcd for $C_{35}H_{47}N_7O_8 \cdot 1.75CH_3CO_2H$: C, 57.88; H, 6.81; N, 12.27. Found: C, 57.84; H, 6.92; N, 12.64.

General Procedure for the Preparation of Urea-Substituted Tetrapeptides (1). Boc-Trp-Lys(N^{ϵ}-3-(methoxyphenylamino)carbonyl)-Asp-Phe-NH_2 (1e). A solution of tetrapeptide **4** (85 mg, 0.12 mmol), phenyl isocyanate (25 μL, 0.18 mmol), and *N*-methylmorpholine (35 μL, 0.32 mmol) in DMF (3 mL) was stirred at ambient temperature for 18 h. The DMF was evaporated in vacuo and the residue directly applied to a silica gel column and eluted with ethyl acetate−pyridine−acetic acid−water (300:20:6:11). After evaporation of solvents, the residue was dissolved in water−acetone (10:1, v:v), lyophilized, and further dried (50 °C) to yield **1e** (29 mg, 39%) as a white solid: MS (FAB^+) *m/e* 843 $(M + H)^+$; ^1H NMR (DMSO-d_6, 300 MHz) δ 1.05−1.68 (m, 4H), 1.30 (br s, 9H), 2.32−3.18 (m, 10H), 3.68 (s, 3H), 4.11−4.51 (m, 4H), 6.41 (d, $J = 7$ Hz, 1H), 6.81 (d, $J = 7$ Hz, 2H), 6.84−7.28 (m, 16H), 7.56 (br d, $J = 7$ Hz, 1H), 7.89 (d, $J = 7$ Hz,1H), 8.00 (m, 1H), 8.16 (m, 1H), 10.82 (br s, 1H).

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