Binding of Norbinaltorphimine (norBNI) Congeners to Wild-Type and Mutant Mu and Kappa Opioid Receptors: Molecular Recognition Loci for the Pharmacophore and Address Components of Kappa Antagonists

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Molecular modifications of both the kappa opioid antagonist norbinaltorphimine (norBNI, **1**) and the kappa receptor have provided evidence that the selectivity of this ligand is conferred through ionic interaction if its N17′ protonated amine group (an "address") with a nonconserved acidic residue (Glu297) on the kappa receptor. In the present study, we have examined the effect of structural modifications on the affinity of norBNI analogues for wild-type and mutant kappa and mu opioid receptors expressed in COS-7 cells. Compounds **2**, **3**, and **7**, which have an antagonist pharmacophore and basic N17′ group in common with norBNI, retained high affinity for the wild-type kappa but exhibited greatly reduced affinity for mutant kappa receptors (E297K and E297A). Modification of the phenolic or N-substituent groups of the antagonist pharmacophore (**4** and **5**) or removal of basicity at the address N17′ center (**6**) led to greatly reduced affinity for the wild-type and mutant receptors. The reduced affinity upon modification of the kappa receptor is consistent with the ionic interaction of the protonated N17′ group of kappa antagonists (**1**−**3**, **7**) with the carboxylate group of E297 at the top of TM6. This was supported by the greatly enhanced affinity of compounds **1**−**3** for the mutant mu receptor (K303E), as compared to the wild-type mu receptor, given that residue K303 occupies a position equivalent to that of E297 in the kappa receptor. In view of the high degree of homology of the seven TM domains of the kappa and mu opioid receptors, it is suggested that the antagonist pharmacophore is bound within this highly conserved region of the kappa or mutant mu receptor and that an anionic residue at the top of TM6 (E297 or K303E, respectively) provides additional binding affinity.

Introduction

The prototypical kappa opioid receptor antagonist, norbinaltorphimine, **1** (norBNI), contains two basic nitrogens, N17 and N17′.[1,2] The N17 basic nitrogen is associated with the antagonist pharmacophore, whereas N17′ was proposed to function as an "address" in conferring kappa selectivity.[3] It was proposed that the "address" recognition locus on the kappa receptor contains a uniquely positioned acidic residue that ion-pairs with N17′. Support for this was obtained from structure−activity relationship (SAR) studies which demonstrated that the second pharmacophore is not necessary for the kappa antagonist activity of norBNI and that the N17′ basic nitrogen (the "address") is a key requirement for selectivity.[4,5] The finding that amidation of N17′ destroys kappa antagonist activity was consistent with this proposal.[5,6] Subsequent studies on mutant and chimeric kappa−mu opioid receptors revealed this residue to be Glu297 (E297) which is located at the top of transmembrane helix 6 (TM6).[7]

Here we present data on the binding of norBNI-related ligands (**2**−**7**) to cloned kappa and mu receptors that have been mutated at position 297 (E297X, X = K or A) in the former and at an equivalent position, K303E, in the latter. The results support the proposal that a glutamate residue (E297) in the wild-type kappa receptor or in the mutant (K303E) mu receptor interacts with the protonated N17′ group of norBNI and its potent analogues. Moreover, the data suggest that the kappa and mutant mu receptor environments surrounding the bound antagonist pharmacophore of norBNI are similar.[8]

Chemistry

The synthesis and biological activities of compounds **1**−**4** and **6** have been reported earlier.[3,6] Compound **5** was prepared by first forming the 3-*O*-methyl ether **9** by treatment of naltrexone (**8**) with diazomethane (Scheme 1) and then reacting **9** with excess oxymorphone (**10**) in the presence of hydrazine under acidic conditions. Excess oxymorphone helped to minimize formation of the 3-*O*-methyl ether dimer.

Compound **7** was obtained by reaction of the corresponding 17′-desalkyl analogue **11**[5] (Scheme 2) with bisBOC-thiopseudourea and mercury(II) chloride using Kim's guanidylation procedure,[9] followed by removal of the BOC groups with trifluoroacetic acid.

Opioid Receptor Binding Affinity

Receptor binding was carried out on whole transfected COS-7 cells. The effect of exchanging key amino acid residues in cloned kappa (E297X) and mu (K303E)

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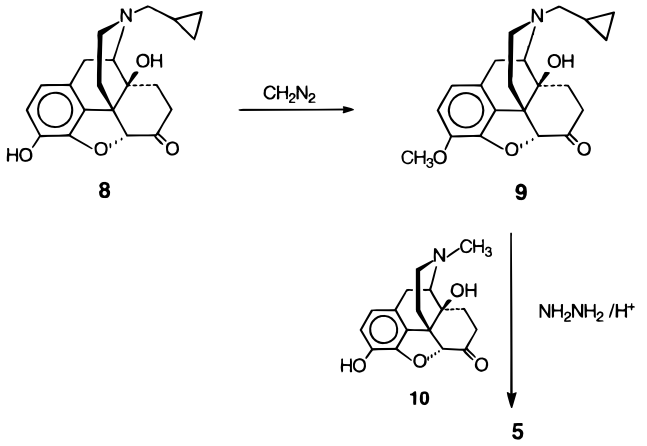
Scheme 1



opioid receptors on the binding affinity of norBNI analogues is presented in Table 1. Among these compounds, norBNI (**1**) showed the highest binding affinity for the wild-type kappa receptor and exhibited a 100-fold loss of binding affinity for both mutant receptors compared to that for the wild type. Compounds **2**, **3**, and **7**, which also possess an antagonist pharmacophore unit in their structures, exhibited binding affinity comparable to that of **1** for the kappa wild-type receptor and also showed a similar degree of loss in mutant kappa receptor binding affinity. On the other hand, compound **6**, which possesses the same antagonist pharmacophore moiety as **1** but differs by an amide group at N17′ (versus the basic character at N17′ for the other compounds), exhibited a >100-fold *less* binding affinity for the wild-type kappa receptor.

The importance of the *N*-cyclopropyl and phenolic functions to the antagonist activity of bimorphinans, as reported earlier[3] in functional assays, is complemented in the present study. Compound **5**, which contains a methoxy function in its antagonist pharmacophore, exhibited a 100-fold reduction in binding affinity for the kappa wild-type receptor.

The binding of **1**−**6** on the wild-type mu receptor was uniformly low. However, the affinity of compounds **1**−**3** for the mutant mu receptor (K303E) was increased by a factor of 100−200-fold over that for the wild-type mu receptor. The difference was similar to that observed between the wild-type and mutant κ opioid receptors.

Discussion

The binding data from the present study complements published functional SAR studies of norBNI analogues.[3−6] The substituent attached to N17′ does not appear to be critical as long as basicity is maintained. Thus, ligands with 17′-cyclopropylmethyl, -methyl, and -amidine groups (**2**, **3**, **7**) all retain high affinity for the wild-type kappa receptor. On the other hand, since the acetyl group eliminates the basicity of N17′, it greatly reduces the affinity of **6**, presumably because such a modification eliminates counterionic attraction for E297.

The finding that the mutant kappa receptors (E297K and E297A) display greatly reduced affinity for norBNI (**1**) and its analogues (**2**, **3**, **7**) that contain a basic N17′ group is consistent with the participation of counterions for high-affinity binding. This is due to the fact that lysine and alanine are unable to interact with N17′ as counterions. Thus, the cationic "address" (protonated N17′) of these ligands and the anionic carboxylate group (E297) of the kappa receptor possibly may confer affinity for the kappa receptor through the formation of a salt bridge.

The fact that E297 is not conserved among mu and delta receptors appears to be the basis for the kappa selectivity of norBNI, inasmuch as the equivalent position in mu and delta opioid receptors contains lysine (K303) and tryptophan (W284) residues, respectively.[10] In this regard, it is likely that the protonated amino group of K303 inhibits binding of norBNI through ionic repulsion of the protonated N17′ group in the active kappa antagonists. The bulky, hydrophobic tryptophan residue (W284) in the delta receptor may inhibit binding through steric repulsion.

The role of E297 as an anionic residue that interacts with the cationic "address" of kappa antagonists has received added support from studies with the mu receptor mutant whose lysine residue was replaced by glutamate (K303E).[7] In this mutant, the glutamate residue is located in a position equivalent to that of E297 in the kappa receptor. The mutant mu receptor displayed greatly enhanced affinity for **1**−**3** relative to the wild-type mu receptor, again highlighting the critical role of E297 in the kappa receptor. What is more, the data suggest that similar binding domains of the kappa and mutant mu receptors recognize the antagonist pharmacophore of the high-affinity ligands (**1**−**3**).[7] This supports the idea that the recognition locus for the pharmacophore is in the central cavity created by the seven TM helices, as they have the greatest homology compared to potential extracellular binding sites on the receptor.[8]

It can be noted that ligands (**4**, **5**) with an "address", but without an accompanying antagonist pharmacophore, possess low affinity for the kappa receptor. Since the phenolic OH and the *N*17-cyclopropylmethyl group are required for recognition of the pharmacophore, their absence in **4** and **5** leads to the >200-fold loss of affinity

Scheme 2

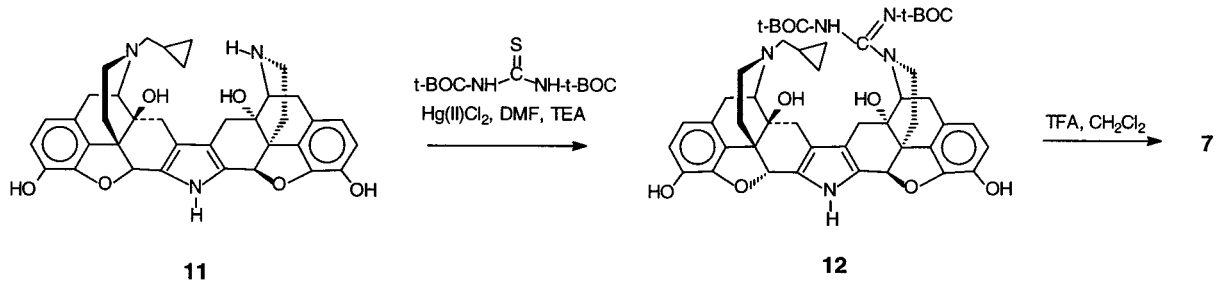
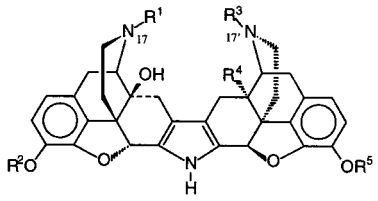


Table 1. Effect of Site-Directed Mutagenesis on Binding to Kappa and Mu Opioid Receptors



						K_i[a] (nM)				
compd	R^1	R^2	R^3	R^4	R^5	wtκ	(E297K)κ	(E297A)κ	wtμ	(K303E)μ
1[b]	$CH_2CH(CH_2)_2$	H	$CH_2CH(CH_2)_2$	OH	H	0.13 ± 0.04 (10)	12.9 ± 1.7 (9)	12.8 ± 1.7 (9)	70.0 ± 17.9 (4)	0.94 ± 0.09 (10)
2[b]	$CH_2CH(CH_2)_2$	H	CH_3	H	H	0.32 ± 0.04 (12)	39.9 ± 6.2 (4)	39.6 ± 6.2 (4)	40.0 ± 5.5 (4)	0.16 ± 0.07 (3)
3[b]	$CH_2CH(CH_2)_2$	H	$CH_2CH(CH_2)_2$	OH	CH_3	0.39 ± 0.03 (11)	28 ± 2.6 (4)	17 ± 10.6 (4)	276 ± 32.3 (2)	2.7 ± 0.3 (3)
4[b]	CH_3	H	CH_3	OH	H	21.7 ± 4.8 (10)	>1000 (4)	>1000 (4)	>1000 (4)	41.7 ± 10.4 (4)
5	$CH_2CH(CH_2)_2$	CH_3	CH_3	OH	H	36.3 ± 3.0 (8)	>1000 (3)	>1000 (2)	165 ± 2.3 (4)	52 ± 19 (4)
6[c]	$CH_2CH(CH_2)_2$	H	$COCH_3$	OH	H	23.6 ± 4.6 (9)	104 ± 8.4 (4)	104 ± 8.4 (4)	102 ± 27.5 (4)	42 ± 4.8 (3)
7	$CH_2CH(CH_2)_2$	H	$(C{=}NH)NH_2$	OH	H	0.57 (1)	189 (2)			
diprenorphine						0.28 ± 0.05 (11)	0.37 ± 0.05 (16)	0.38 ± 0.05 (16)	0.27 ± 0.05 (10)	0.33 ± 0.10 (12)

[a] K_i values were determined from competition binding curves using [^{3}H]diprenorphine as the radioligand and analyzed on intact transfected COS-7 cells in the same manner as described previously.[7] Column headings, such as "wtκ", refer to the wild-type kappa receptor preparation, and "(E297K)κ" relates to the preparation resulting from mutagenic exchange of glutamic acid residue 297 for lysine of the kappa receptor. Data are presented as mean ± standard error with the number of replicate experiments indicated in parentheses. [b] Preparation and smooth muscle pharmacology described in ref 3. [c] Preparation and smooth muscle pharmacology described in ref 5.

relative to norBNI. Consequently, it is the combined affinity of the pharmacophore and the "address" that contributes to the high affinity of norBNI and other potent kappa antagonists in the series.

Conclusion

Molecular modification of both norBNI and opioid receptors has led to greater insight into the molecular recognition of norBNI and its analogues by kappa opioid receptors. The "address" (N17′) of the ligands must be sufficiently basic to be in the protonated state at physiologic pH in order for it to associate with the carboxylate group of E297 in the kappa receptor. The protonated N17′ address is rigidly held in a specific orientation by a scaffold which directs it to E297 when the ligand is bound to the kappa receptor.[11,12] In this regard, the geometry and length of the scaffold are important for bringing the counterions together, as it has been reported that a nonlinear scaffold leads to greatly reduced kappa antagonist potency.[11] Using norBNI as a template, the distance between the N17 and N17′ basic groups is ∼11 Å, and this approximately matches the distance between the conserved aspartate residue in TM3 (D138) and the nonconserved glutamate (E297) at the top of TM6[13] (Figure 1). This correspondence suggests that the cationic groups of norBNI and its potent analogues are proximal to D138 and E297 when bound in the central cavity of the kappa receptor. Given that the seven-TM domain of kappa and mu receptors possesses a high degree of homology,[14] and the finding that the mutant mu receptor (K303E) binds kappa antagonists with high affinity, it appears likely that the antagonist pharmacophore is bound within this highly conserved region of the kappa receptor.

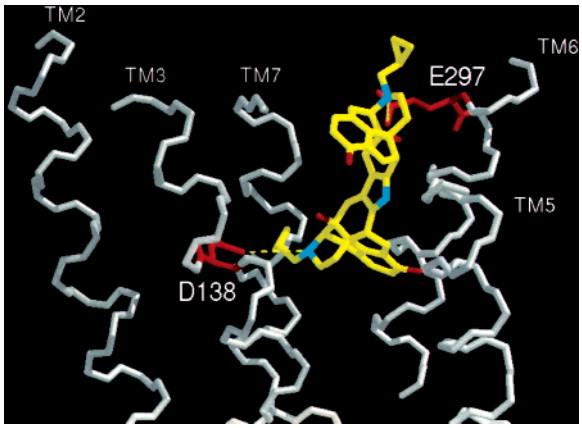


Figure 1. Model of norBNI (**1**) docked to its recognition site on the wild-type kappa receptor. Note the association of N17 and N17′ with residues D138 in TM3 and E297 in TM6, respectively. Transmembrane helices TM1 and TM4 have been deleted to aid visualization.

Experimental Section

All reagents were obtained from the Aldrich Chemical Co. unless stated otherwise. Naltrexone was obtained from Mallinckrodt. All reactions described were conducted in an inert atmosphere of argon or nitrogen unless otherwise stated. Column chromatography was performed using silica gel (200−400 mesh; Aldrich Chemical Co.) as the stationary phase and nitrogen pressure. Thin-layer chromatography was performed on silica gel 60 F_{254} 0.25-nm aluminum-backed sheets (E. Merck) and visualized with UV light, phosphomolybdic acid, or iodine vapor. Chromatographic elution solvent systems are reported as volume/volume ratios. IR spectra were recorded on a Perkin-Elmer PE-281 spectrophotometer or a Nicolet 5DXC FT-IR spectrometer using KBr disks. NMR spectra were obtained using a Varian Unity 300 MHz or Varian Inova 300 MHz instrument at room temperature and $CDCl_3$, CD_3OD, or $(CD_3)_2S{=}O$ as solvents. The δ scale (ppm) was in reference to the deuterated solvent, and coupling constants (J) are reported in hertz (Hz). Mass spectra (FAB) were obtained on a VG-707EHF spectrometer using a *m*-nitrobenzyl alcohol (MNOBA) matrix or (EI) on a Finnigan MAT 95 instrument. The purity of compounds was assessed by analytical HPLC on an Alltech C18, 5-μm, 4.6-mm × 250-mm column using 10−15% aqueous acetonitrile + 0.1% CF_3COOH run isocratically. Melting points were determined in open capillary tubes with a Thomas-Hoover melting point apparatus and are uncorrected.

17-Methyl-17′-cyclopropylmethyl-3′-methoxy-6,6′,7,7′-tetradehydro-4,5α:4′,5′α-diepoxy-6,6′-(imino)[7,7′-bimorphinan]-3,14,14′-triol (5). Naltrexone 3-*O*-methyl ether (**9**)[15] was prepared by reacting naltrexone (315 mg, 1.0 mmol) with

excess diazomethane (generated from 1-methyl-3-nitro-1-nitrosoguanidine and sodium hydroxide in biphasic Et_2O/water) in a mixture of tetrahydrofuran (5 mL) and diethyl ether (5 mL) at ambient temperature. Upon completion of the reaction, the solvent was removed under reduced pressure to afford an oil, which was chromatographed on silica using $CHCl_3-NH_4OH$ (99:1) to afford **9** as an oil: 270 mg, 76%; TLC R_f 0.52 ($CHCl_3-NH_4OH$, 99:1); ^{1}H NMR ($CDCl_3$) δ 6.68 & 6.60 (2d, 2H, J = 8.15 Hz, C1 & C2), 4.66 (s, 1H, C5), 3.88 (s, 3H, OCH_3); EI MS m/z 355 (M^+, 100%); mp 236 °C (hydrochloride salt).

Oxymorphone (**10**) (344 mg, 1.14 mmol) was dissolved in glacial acetic acid (5 mL) and mixed with a solution of **9** (135 mg, 0.38 mmol) in glacial acetic acid (5 mL). Hydrazine dihydrochloride (80 mg, 0.76 mmol) was added, and the reaction mixture was stirred at 90 °C for 24 h and for an additional 24 h at 95 °C under a stream of nitrogen. The reaction mixture was cooled, and the volatile components were removed under reduced pressure with the aid of a heptane azeotrope. The oily residue was subjected to column chromatography using an elution gradient of $CHCl_3$−MeOH (0−10%)−NH_4OH (1%) to yield pure **5**: 32 mg (18%); mp (dihydrochloride salt) >260 °C; TLC R_f 0.44 (EtOAc−MeOH−NH_4OH, 90:10:1); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.47 (s, 1H, pyrrole NH), 6.52 (m, 4H, H2, H2′, H1, H1′), 5.41 (s, 1H, H5), 5.37 (s, 1H, H5′), 3.70 (s, 3H, CH_3O), 2.26 (s, 3H, NCH_3), 0.79 (m, 1H, cyclopropane CH), 0.41 (m, 4H, cyclopropane $CH_2\alpha$), 0.12 (m, 4H, cyclopropane $CH_2\beta$); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 144.7 (C3′), 143.3 & 143.0 (C4 & C4′), 139.0 (C3), 130.8 (C12), 130.5 (C12′), 125.6 (C11), 125.1 (C11′), 124.6 (C6 & C6′), 118.7 (C1), 118.1 (C1′), 117.3 (C2), 116.2 (C7), 116.1 (C7′), 113.2 (C2′), 85.8 (C5), 85.2 (C5′), 73.1 (C14), 72.8 (C14′), 65.0 (C9), 62.4 (C9′), 59.4 (C18 & C18′), 56.1 (CH_3O), 47.8 (C13), 47.4 (C13′), 45.4 (C16), 43.7 (C16′), 42.9 (NCH_3), 31.6, 31.3 (C8 & C8′), 28.9, 28.8 (C10 & C10′), 23.1, 22.4 (C15 & C15′), 7.5 (C19 & C19′), 4.0 (C20 & C20′), 3.8 (C21 & C21′); IR (KBr) cm^{-1} 3416 (s), 1636 (m), 1506 (m), 1457 (w), 1328 (w), 1121 (m), 1046 (m); HRMS (FAB) m/z calcd for $C_{38}H_{41}N_3O_6$ $(M + H)^+$ 636.3074, obsd 636.3088.

17-Cyclopropylmethyl-17′-guanidinyl-6,6′,7,7′-tetradehydro-4,5α:4′,5′α-diepoxy-6,6′-(imino)[7,7′-bimorphinan]-3′,3,14,14′-tetrol (7). 17-Cyclopropylmethyl-6,6′,7,7′-tetradehydro-4,5α:4′,5′α-diepoxy-6,6′-(imino)[7,7′-bimorphinan]-3′,3,14,14′-tetrol[5] (**11**) (73.4 mg, 0.12 mmol) was dissolved in anhydrous dimethylformamide (3.0 mL) and cooled to 0 °C in an ice bath. 1,3-Bis(*tert*-butoxycarbonyl)-2-methylthiopseudourea (36 mg, 0.13 mmol) and triethylamine (0.052 mL, 0.37 mmol) were added sequentially, and the reaction mixture was allowed to stir for 10 min at 0 °C. Mercury(II) chloride (1.1 equiv, 35 mg) was added in one portion, and rapid stirring was maintained for 20 min, after which the ice bath was removed and the reaction mixture was allowed to attain ambient temperature over 1 h. The mixture was filtered through a Celite pad under vacuum to remove mercuric sulfide, and the pad was subsequently washed repeatedly with methanol. Removal of all volatile components under reduced pressure produced an oil, which was subjected to flash column chromatography using $CHCl_3$−MeOH−NH_4OH (80:10:1) to afford the *N*17′-bisBOC-guanidinyl derivative **12** as a white crystalline solid: yield 74 mg (73%); ^{1}H NMR (CD_3OD, 300 MHz) δ 6.49 (m, H1, H2, H1′, H2′), 5.45 (s, 1H, H5), 5.42 (s, 1H, H5′), 4.17 (d, 1H, J = 7.0 Hz, H9′), 3.71 (dd, 1H, H9, J = 4.7 Hz, J = 14.5 Hz), 3.11−3.42 (m, 5H), 2.61−3.11 (m, 5H), 2.20−2.61 (m, 6H), 1.73 (m, 1H, H15), 1.60 (m, 1H, H15′), 1.43 (s, 9H, t-BOC), 1.41 (s, 9H, t-BOC), 0.96 (m, 1H, H19), 0.60 (m, H20α & H21α), 0.29 (m, H20β & H21β); HRMS (FAB) m/z (%) calcd for $C_{47}H_{58}N_5O_{10}$ $(M + H)^+$ 853.4262, obsd 853.3829.

Removal of the BOC groups was accomplished by dissolving **12** (50 mg, 0.05 mmol) in anhydrous dichloromethane (4 mL) under nitrogen, cooling the solution to 0 °C, and then adding trifluoroacetic acid (1 mL) dropwise over a 10-min period. The reaction mixture was allowed to stir at ambient temperature for a further 48 h, and then the volatile components were removed under reduced pressure and the resultant oil was washed with diethyl ether. The residual white precipitate was isolated by filtration and purified by RP-HPLC (Alltech C18, 10 μm, 22 mm × 250 mm; water−acetonitrile−TFA, 90:10:0.25, isocratic) to afford **7**·2CF_3COOH as an amorphous solid: yield 15 mg (34%); mp >230 °C; ^{1}H NMR (CD_3OD, 300 MHz) δ 6.49 (m, H1, H2, H1′, H2′), 5.45 (s, 1H, H5), 5.42 (s, 1H, H5′), 4.17 (d, 1H, J = 7.0 Hz, H9′), 3.71 (dd, 1H, H9, J = 4.7 Hz, J = 14.5 Hz), 3.11−3.42 (m, 5H), 2.61−3.11 (m, 5H), 2.20−2.61 (m, 6H), 1.73 (m, 1H, H15), 1.60 (m, 1H, H15′), 0.96 (m, 1H, H19), 0.60 (m, H_2O & H21), 0.29 (m, H20 & H21); ^{13}C NMR (CD_3OD, 75.4 MHz) δ 159.44, 145.04, 144.76, 141.56, 141.12, 132.27, 131.81, 131.63, 126.66, 126.40, 126.24, 126.01, 125.04, 124.83, 119.91, 119.77, 119.50, 118.44, 117.94, 116.70, 116.17, 86.18, 85.91, 74.67, 74.63, 74.48, 63.52, 61.01, 60.46, 55.18, 44.95, 41.11, 35.94, 33.64, 33.41, 32.72, 30.84, 30.45, 29.94, 24.05, 21.85, 10.19, 4.62, 4.14; HRMS (FAB) m/z (%) calcd for $C_{37}H_{39}N_5O_6$ $(M + H)^+$ 650.2978, obsd 650.2849.

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