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Synthesis of iboga-like isoquinuclidines: Dual opioid receptors agonists having antinociceptive properties

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ABSTRACT

Some novel iboga-analogues consisting of benzofuran moiety and dehydroisoquinuclidine ring connected by $-CH_2-$, $(CH_2)_2$ and $(CH_2)_3$ linkers have been synthesized with the view to develop potential antinociceptive drugs. The compounds **14** and **21** showed binding at the μ-opioid receptor (MOR), while the compound **11a** exhibited dual affinities at both MOR and κ-opioid receptor (KOR). MAP kinase activation indicated all three compounds have opioid agonistic properties. The presence of a double bond and *endo*-methylcarboxylate group in the dehydroisoquinuclidine ring and the benzofuran and methylene spacer appeared to be essential for opioid receptor binding. Further studies demonstrated **11a** caused significant antinociception in mice in the hot-plate test which was comparable to that produced by morphine. The compound **11a** was also found to be nontremorigenic unlike various iboga congeners. This study identifies a new pharmacophore which may lead to the development of suitable substitute of morphine in the treatment of pain.

1. Introduction

Opioids exert their activity through their interaction with the μ-(MOR), κ-(KOR) or δ-(DOR) opioid receptors. Morphine is a MOR agonist, which has been used extensively for the treatment of severe pain for several decades but produces serious side effects like tolerance, dependence, withdrawal symptoms, respiratory depression, decreased gastric motility, and emesis.[1–3] One approach to limit μ-receptor-mediated side effects was to selectively target KOR and DOR. The function of DOR is less understood, but KOR has been receiving much research interest because of its functional interaction with the μ-receptor. It has been shown that KOR agonists produce analgesia both in animals and in humans, although it appears that they are not as powerful as μ-opioids in producing antinociception, at least in some forms of pain.[4–6] Previous studies from several research groups have demonstrated that selective KOR agonists, such as U50488, U69593 and CI977, or the less-selective endogenous KOR agonist dynorphin, produce antinociception in several forms of analgesic tests.[7–9] However, in other studies similar doses of these agonists failed to modulate pain threshold in comparable tests. The KOR agonists are limited to their use as peripheral analgesics owing to their psychotomimetic and dysphoric central effects. In this context, an alternative approach that has gained credence was the development of compounds, from natural sources or through synthetic means, which possess opioid activity at the different opioid receptors.[10,3] One such compound was ibogaine (Fig. 1), an alkaloid extracted from *Tabernanthe iboga*. Although it was originally reported to possess high affinity for KOR with no affinity for MOR or DOR,[11,12] subsequent studies showed that ibogaine possessed affinity for the MOR and iboga derivatives like noribogaine and 18-methoxycoronaridine, which are potentially non-toxic, were more active than ibogaine at both MOR and KOR.[13,14] Despite having potential opioidergic activity and anti-addictive properties, ibogaine exhibits several side effects such as hallucinations, tremors, ataxia[15] and degeneration of cerebellar purkinje cells.[15,16] Problems associated with ibogaine led to an attempt to develop a safer and still efficacious structural derivative. There have been few attempts in the past to study the structure–activity relationship of various ibogaine congeners which established a correlation of tremorigenic activity with the indole moiety of the congeners.[17] Studies with other ibogaine congeners demonstrated that most of the compounds showed tremorigenic activity to some extent, except, the compound, 18-methoxycoronaridine (18-MC) which was devoid of any such toxicity. Interestingly, unlike ibogaine, 18-MC has no affinity at the NMDA receptors or the serotonin transporter.[18] Moreover, MC bound to all three opioid receptors (κ, μ, and δ) as

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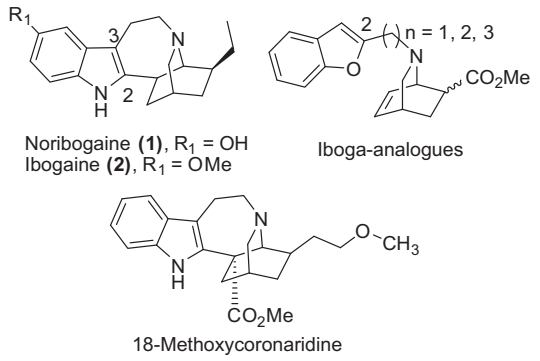


Figure 1. Ibogaine, noribogaine and iboga-analogues.

well as to the 5-HT3 receptor, having comparatively greater affinity for the MOR compared to the other congeners.

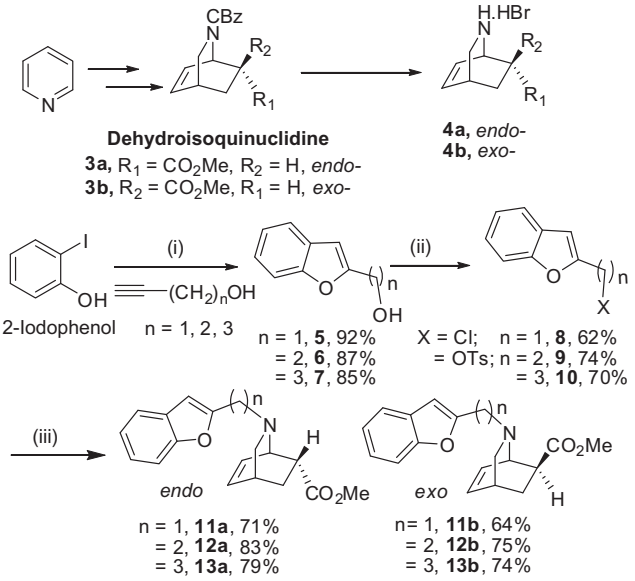
All the members of iboga family combine the structural features of indole and isoquinuclidinyl ring fused by a seven-membered indoloazepine ring. Interestingly, scientists have already synthesized tropane-3-indoles[12] and hexahydroazepino-benzothiophenes[19] as abbreviated ibogaine analogues which have been found to recognize many of the same molecular targets as ibogaine. It has also been reported that the absence of the azepine ring does not seem to limit a considerable affinity for the receptorial targets of ibogaine.[20] The 2-azabicyclo[2.2.2]octane (or isoquinuclidine) framework is a distinctive motif in a variety of both naturally occurring[21,22] and pharmaceutical[23,24] products and its structural feature is currently encouraging the development of synthetic methods.[25,26] In this connection our present study aims towards the synthesis, characterization and biological activities of iboga-analogues, without having the azepine ring and replacing the indole ring with a benzofuran moiety (Fig. 1).

The selection of benzofuran moiety over indole was on the basis of the following reasons. Benzofurans are expected to be more stable than the natural ibogaine since the latter is heat and light sensitive and spontaneously oxidized in solution. Moreover, the synthesis of benzofuran could be easier than indole as it does not require any protection like indole. Being a bioisostere of indole, the pharmacological properties of benzofuran moiety should remain unchanged, since the NH group of indole does not appear to be important for receptor binding activities.

2. Results and discussion

2.1. Chemistry

Benzofuran isoquinuclidine fused compounds can be synthesized easily from the coupling of 2-substituted benzofuran alcohols and dehydroisoquinuclidine ring as shown in Scheme 1. The common fragment, dehydroisoquinuclidine was synthesized using Diels–Alder reaction following our published protocol.[27,28] Both isomers **3a** and **3b** were separated using alumina column chromatography and used for further reactions. For the synthesis of iboga-analogues, the requisite 2-benzofuran alcohols were synthesized in one pot from 2-iodophenol via Sonogashira coupling with terminal alkynols at room temperature as shown in Scheme 1. Tosylation of 2-benzofuran alcohols **6**[27] and **7** gave the tosylated products **9** and **10**, respectively. Interestingly, we isolated the chlorinated product **8** in case of compound **5** during tosylation. Conjugation with **4a** or **4b** afforded 2-substituted benzofuran isoquinuclidine derivatives **11**, **12** and **13** in overall good yields. After first phase screening with six compounds, compound **11a** was found to be the most potent and based on the structure following compounds were



Scheme 1. Synthesis of dehydroisoquinuclidine and its iboga-analogues; Reagents and conditions: (i) $Pd(OAc)_2$, PPh_3, CuI, Et_3N, rt; (ii) TsCl, Et_3N, CH_2Cl_2, 0 °C to rt; (iii) **4a** or **4b**, K_2CO_3, CH_3CN, 80 °C.

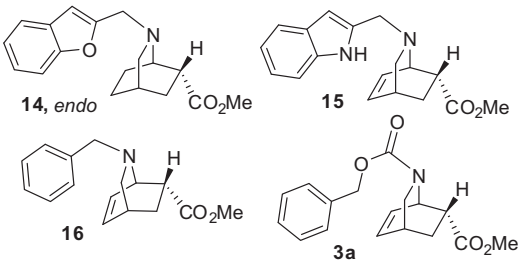


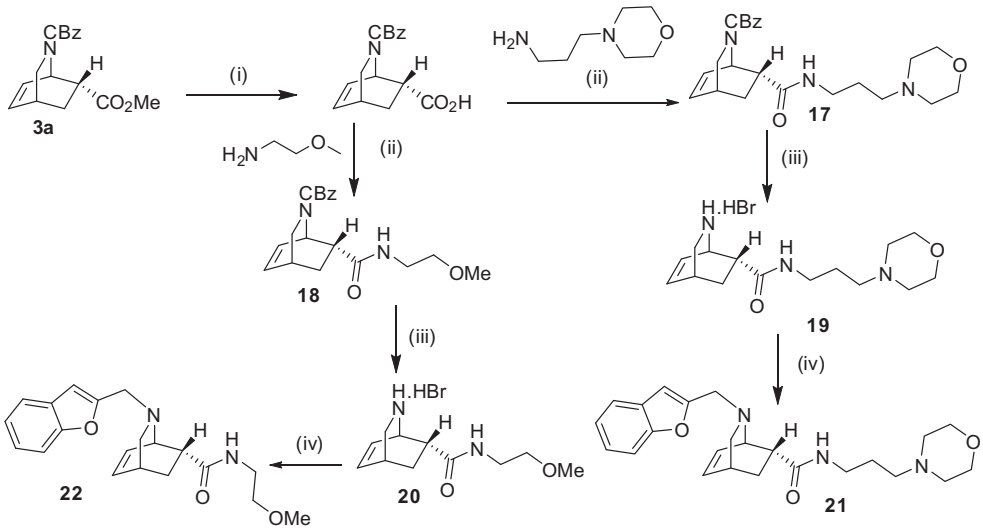
Figure 2. Compounds synthesized based on the structure of compound **11a**.

synthesized and screened (Fig. 2). Compound **14** was synthesized from compound **11a** using Pd–C/H_2 as a reducing agent to check the role of the double bond. To explore the role of benzofuran ring, compounds **15** and **16** were screened along with compound **3a**. Compound **15** was synthesized following our lab protocol.[28]

To find the role of C-7 methylcarboxylate group, compounds **21** and **22** were synthesized using the protocol as shown in Scheme 2. *endo*-Isoquinuclidine carboxylic acid was conjugated separately with 3-morpholinopropan-1-amine and 2-methoxyethylamine using EDCI coupling to obtain compounds **17** and **18**, respectively. These two groups were chosen because they are pharmacologically important and used in many medicinally important compounds. Another reason to choose 2-methoxyethyl chain was due to its presence in 18-methoxycoronaridine, which had activity at the opioid receptors. Deprotection of benzyloxycarbonyl, followed by conjugation with **8**, gave us compounds **21** and **22**, respectively.

2.2. Biology

We further investigated the effect of these novel iboga-analogues on the opioid receptor subtypes using in vitro binding studies. Displacement of specific [^{125}I]-DAMGO binding to evaluate the interactions at MOR showed that some of the compounds effectively displaced [^{125}I]-DAMGO from the membrane receptors, the order of affinity being **21** > **14** > **11a**, the others being inactive (Supplementary material, Table S1).



Scheme 2. C-7 modification of compound **11a**; Reagents and conditions: (i) LiOH, MeOH; (ii) EDCI, HOBT, CH_2Cl_2; (iii) 20% HBr–AcOH; (iv) **8**, K_2CO_3, CH_3CN.

On the other hand, displacement of specific [^{125}I]-dynorphin binding showed that except **11a**, none of the test compounds showed any appreciable binding at the KOR. None of the compounds had any appreciable effect in displacing specific [^{125}I]-DADLE binding at DOR. Overall, the binding studies demonstrated that only **11a** had high affinities for both MOR and KOR, which were comparable to that of selective ligands like DAMGO and U50488H for their respective receptors.

Affinity studies for the interaction of **11a**, **14** and **21** at the MOR were evaluated by calculating the IC_{50} values (Fig. 3). It was observed that all the three compounds had high affinity for MOR with an IC_{50} of 1.61, 0.142 and 0.575 μM, respectively. Similarly the binding affinity of **11a** with KOR was calculated with respect to the KOR-selective ligand U50488H and the IC_{50} values were 2.729 and 1.584, respectively (Supplementary material, Fig. S1).

Both KOR and MOR agonists and not antagonists cause transient activation on pERK[29] in cultured cells within a few minutes of application. Based on the above observations, our laboratory had previously developed an assay, using cultures of C6 glioma, to determine the opioid agonistic or antagonistic property of the test compounds.[30,31]

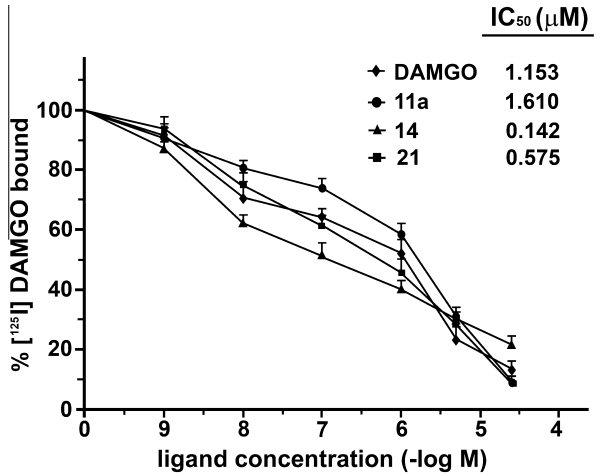


Figure 3. Competitive inhibition of specific [^{125}I]-DAMGO binding to brain membrane by **11a**, **14** and **21**. Corresponding displacement curves by selective MOR agonists, DAMGO was also carried out for comparison. Each point represents mean of three individual determinations. IC_{50} concentrations were determined.

Western blotting analysis, demonstrated that compounds **11a**, **14** and **21** stimulated ERK phosphorylation in C6 glioma cells like the κ-opioid ligand U50488H (Fig. 4a and b). Moreover, stimulation of pERK1/2 level by these test compounds could be blocked by co-incubation with the opioid receptor antagonist naloxone (10 μM). None of the other compounds tested, stimulated ERK phosphorylation to a significant extent (Supplementary material, Fig. S2).

From this study we have optimized a structural requirement from a class of novel iboga-analogues possessing two moieties, a 2-azabicyclooctane (dehydroisoquinuclidine) ring and a benzofuran moiety connected by a short linker chain (methylene group) and identified a compound, **11a** having dual affinities to MOR and KOR.

Interestingly, unlike **11a** which has the methylcarboxylate group at the *endo* position of the isoquinuclidine ring, the compounds **11b**, **12b** or **13b**, which have methylcarboxylate group in the *exo* position, showed loss of activities at all the receptor subtypes. Substitutions of the methylcarboxylate group with an amido group attached to a morpholine in the compound **21** and a methoxy (OMe) group in the compound **22**, caused loss of affinity for KOR. Replacement of the benzofuran group by other moieties in the compounds, **15**, **16** and **3a**, also caused loss of affinities for the opioid receptors, suggesting the importance of benzofuran structure for opioid activity. Length of the linker chain appeared to contribute to the binding affinities as increased chain length in **12a**, **13a** caused loss of activities. Interestingly, unlike **11a**, absence of the double bond between 5th and 6th position in the dehydroisoquinuclidine ring resulted in loss of affinity for KOR as observed for the compound **14** although its activity at the MOR was retained. In this study, **11a** exhibited high affinities for MOR and KOR.

Pain sensation is a very complex phenomenon, and several neuronal systems and transmitters are involved in nociception where opioidergic systems play important and complex roles in the modulation of nociception. Opioid-induced analgesia is due to actions at several sites in the central nervous system. Morphine and other MOR agonists selectively inhibit various nociceptive reflexes, but other sensory modalities remain unaffected.[32]

Profound analgesia can also be produced by supra-spinal mechanism, most notably by effects mediated through the periaqueductal gray matter, the nucleus raphe magnus and the locus ceruleus.

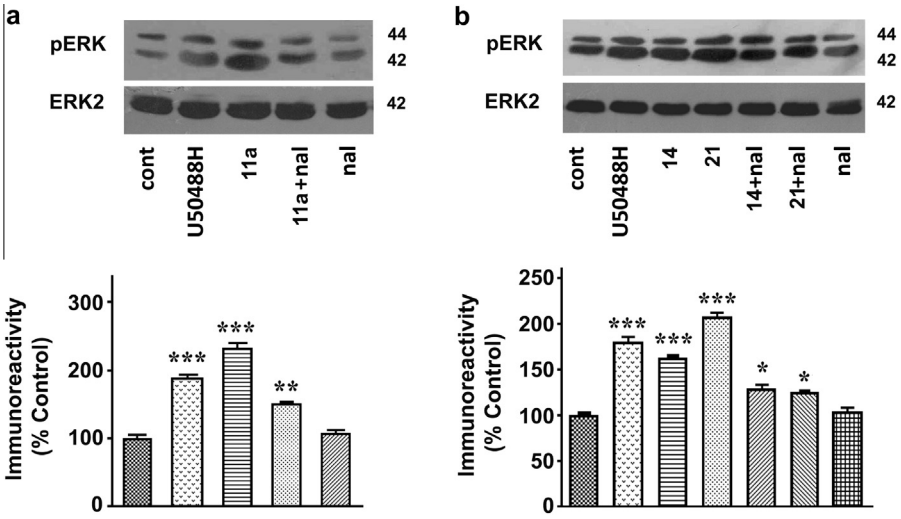


Figure 4. Effect of test compounds on induction of pERK activity in C6 glioma cells. Five-days old cultures were serum starved for 24 h. The stimulatory effect of opioid agonist such as κ-opioid receptor agonist, U50488H (1 μM) and the test compounds (40 μM) were evaluated by treating cells for 10 min with the compounds and quantitating pERK1/2 by Western blot analysis. (a) Shows stimulation of pERK by **11a** and (b) by **14** and **21** and were antagonized by co-treatment with naloxone (10 μM). Same blots were probed with ERK2 which served as a loading control. The relative intensities of the pERK bands, indicated in the graph, were obtained by densitometric scanning and pERK levels, quantitated after normalizing against ERK2. Results are mean ± SEM of at least 3 blots. $^{*}p$ <0.05, $^{**}p$ <0.01 and $^{***}p$ <0.001 versus untreated control.

Opioid receptor activation in these centres results in enhanced activity of the descending aminergic bulbospinal pathways that inhibit the processing of nociceptive information in the spinal cord.[33] Although MOR plays the predominant role in mediating analgesia, it also facilitates the development of tolerance and dependence, unlike KOR. Hence development of drugs having dual agonistic effects at both MOR and KOR could remove the unwanted effects of classical MOR agonists. Various studies have shown mixed KOR/MOR receptor agonists to have lesser side effects than highly selective KOR agonists.[34,35]

Compounds like MCL-101, cyclorphan, benzomorphan etc., having varying affinities for MOR and KOR, has been shown to be effective against drug dependence and drug self administration.[36–38] The analogues and isomers of some of the above compounds were also found to possess antinociceptive properties.[35,39,40] Since the compound **11a** exhibited opioid agonistic properties with potential affinities for both MOR and KOR, it was then selected for evaluation of antinociceptive activity.

Dose–response studies using intraperitoneal (ip) administration of 20, 30 and 40 mg/kg body weight (B.W) of **11a** in mouse hot-plate tests showed a dose related reduction in the mean response time for onset of analgesia, characterized by paw withdrawal in the mice against heat (Fig. 5a). The percentage maximum possible effect (%MPE) of **11a** after 15 min at the three different doses of 20, 30 and 40 mg/kg B.W. were found to be ∼20%, >40% and >80%, respectively while that of morphine (10 mg/kg) and vehicle (DMSO) were >90% and <20%, respectively (Fig. 5b).

Observations of animals treated with morphine (10 mg/kg) and **11a** (30 mg/kg) indicated that there was a significant increase in the maximum possible effect compared to vehicle control group (Fig. 5b). The analgesic effect of **11a** peaked by 15 min at a 40 mg/kg dose which was comparable to morphine treated (10 mg/kg) animals. One important consideration in choosing an analgesic is the duration of its effect. The duration of the maximum analgesic response of morphine at a dose of 10 mg/kg was observed to be 45 min after ip administration in mice. Further,

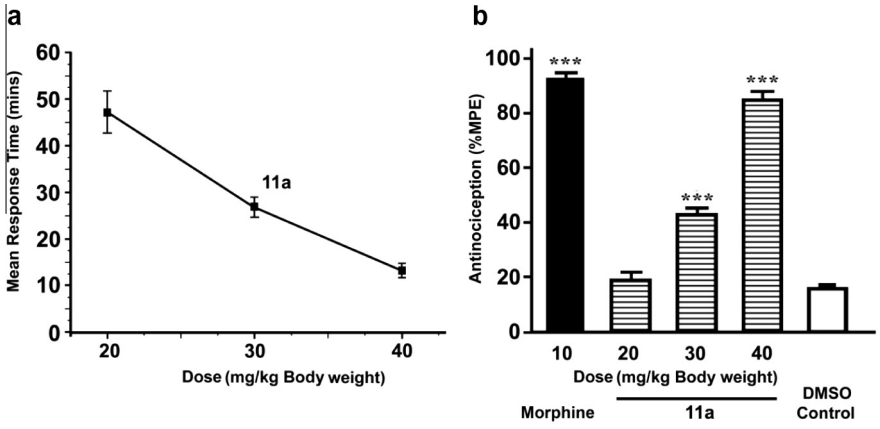


Figure 5. (a) Effect of ip administration of different doses (20, 30 and 40 mg/kg, respectively) of the compound **11a** on mean response time to paw withdrawal in mice. All experiments contained at least 6 animals in each group. (b) Acute effect of different doses (20, 30 and 40 mg/kg, respectively) of the compound **11a** on antinociception score (% maximum possible effect) along with a test dose of morphine (10 mg/kg) in mice. Mice received DMSO as vehicle control. Results are mean ± SEM. Asterisks denote significant differences compared to control group ($^{***}p$ <0.001).

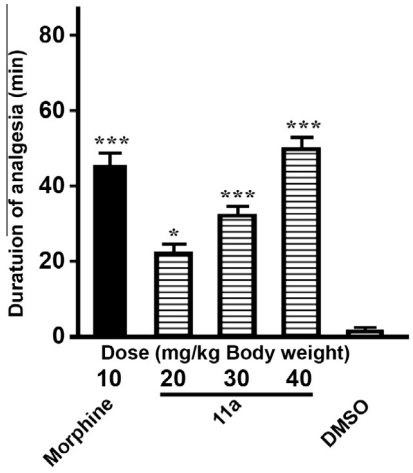


Figure 6. Effect of ip administration of different doses (20, 30 and 40 mg/kg, respectively) of the compound **11a** on duration of analgesic response after the onset of analgesia over times along with a test dose of morphine (10 mg/kg) on a hot-plate in mice. Mice received DMSO as vehicle control. Results are mean ± SEM. Asterisks denote significant differences compared to control group (*p <0.05 and ***p <0.001).

the present study also showed that the animals treated with **11a** (40 mg/kg) exhibited greater duration of analgesia which persisted for more than 50 min unlike that achieved with morphine (Fig. 6).

While ibogaine and its various congeners have been shown to cause tremors in rodent models,[17,41,42] the compound **11a** did not show any demonstrable tremorigenic activity in mice when tested with three different doses, 20, 30, 40 mg/kg B.W., whereas physostigmine at doses of 0.25, 0.5 mg/kg B.W, showed significant tremorigenic activity under the experimental conditions (Supplementary material, Tables S2 and S3).

3. Conclusion

In conclusion, the present study reports, the synthesis of some new iboga-analogues. One of these compounds **11a**, was found to have dual affinities for MOR and KOR. Compound **11a** also possessed potential analgesic property comparable to that of morphine in the hot-plate test in mice model. Further investigations will be carried out to study the possible mechanism of analgesic activity. We also plan to synthesize the compound on a large scale to test whether it causes physical dependence and tolerance in animals.

4. Experimental protocols

4.1. Chemistry

4.1.1. General

Unless noted otherwise, all reactions were conducted under argon and, where appropriate, under anhydrous conditions using commercially available substrates and solvents. A usual workup of the reaction mixture consists of extraction with common organic solvents (ether, ethylacetate, dichloromethane), washing with water, brine, drying over Na_2SO_4, and then concentrated under reduced pressure on a rotary evaporator unless specified. Yields (isolated) were reported after purification of the crude by column chromatography. Coupling constants are provided in Hz, with the following spectral pattern designations: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublets; dt, doublet of triplets; m, multiplet; br, broad; rt, room temperature.

4.1.1.1. Benzofuran-2-ylmethanol (5). To a well stirred mixture of 2-iodophenol (0.50 g, 2.27 mmol), palladium acetate (0.026 g, 0.11 mmol), CuI (0.022 g, 0.11 mmol) and triphenylphosphine (0.003 g, 0.11 mmol) in dry triethylamine (5.0 mL) was added propargyl alcohol (0.14 g, 2.5 mmol) under argon atmosphere. The reaction mixture was then stirred for overnight at room temperature. Reaction mixture was concentrated, diluted with EtOAc (20 mL), the EtOAc layer was washed with H_2O (2 × 10 mL) and brine (10 mL). The organic layer was dried over Na_2SO_4, concentrated in vacuo. After column purification on silica gel (100–200 mesh), the compound (**5**) was obtained as yellow oil. Yield: 0.31 g, 92.0%. R_f (4:1, petroleum ether/EtOAc) = 0.45. IR (neat/$CHCl_3$): ν 3323, 1722, 1450, 1253, 1012, 748 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 2.23 (1H, br s), 4.79 (2H, s), 6.67 (1H, s), 7.22–7.33 (2H, m), 7.48–7.50 (1H, d, J = 8.0 Hz), 7.56–7.59 (1H, d, J = 7.8 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ 58.2, 104.2, 111.4, 121.3, 122.9, 124.5, 128.3, 155.2, 156.5.

4.1.1.2. 2-(Benzofuran-2-yl)ethanol (6). Compound (**6**) was prepared following similar procedure as (**5**). Yield: 87.0%. R_f (4:1, petroleum ether/EtOAc) = 0.43. IR (neat/$CHCl_3$): ν 3365, 1602, 1454, 1251, 1047, 750 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 1.98–2.06 (1H, m), 2.89 (2H, t, J = 7.5 Hz), 3.73 (2H, br s), 6.42 (1H, s), 7.18–7.24 (2H, m), 7.39–7.49 (2H, m); ^{13}C NMR (125 MHz, $CDCl_3$): δ 24.9, 61.9, 102.3, 110.8, 120.3, 122.6, 123.3, 128.9, 154.8, 158.8.

4.1.1.3. 3-(Benzofuran-2-yl)propan-1-ol (7). Compound (**7**) was prepared following similar procedure as (**5**). Yield: 85.0%. R_f (4:1, petroleum ether/EtOAc) = 0.43. IR (neat/$CHCl_3$): ν 3350, 2945, 1732, 1454, 1251, 1047, 750 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 1.88 (1H, br s), 1.99–2.06 (2H, m), 2.87–2.90 (2H, t, J = 7.5 Hz), 3.73 (2H, br s), 6.42 (1H, s), 7.18–7.24 (2H, m), 7.40–7.43 (1H, d, J = 9.0 Hz), 7.47–7.49 (1H, d, J = 9.0 Hz). ^{13}C NMR (125 MHz, $CDCl_3$): δ 24.9, 30.7, 61.9, 102.3, 110.8, 120.3, 122.6, 123.3, 128.9, 154.8, 158.8.

4.1.1.4. 2-(Chloromethyl)benzofuran (8). To a stirred solution of *p*-toluenesulfonyl chloride (0.45 g, 2.7 mmol), triethylamine (0.68 mL, 4.9 mmol) and 4-dimethylaminopyridine (0.04 g, 0.33 mmol) in dry CH_2Cl_2 (5 mL) was added slowly a solution of benzofuran-2-ylmethanol (**5**) (0.292 g, 1.97 mmol) in dry CH_2Cl_2 (5.0 mL) at 0 °C. The reaction mixture was stirred for 1 h at 0 °C and left for overnight at room temperature. The reaction mixture was diluted with CH_2Cl_2 (15 mL). The organic layer was washed with water (2 × 10 mL), brine (10 mL), dried over Na_2SO_4. The crude product was purified by column chromatography on silica gel (100–200 mesh) and obtained the pure compound (**8**) as a colorless liquid. Yield: 0.203 g, 62%. R_f (4:1, petroleum ether/EtOAc) = 0.68. IR (neat, $CHCl_3$): ν 2939, 2854, 1452, 1255, 1109, 956 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 4.64 (2H, s), 6.67 (1H, s), 7.15–7.18 (1H, dd, J = 7.0, 3.0 Hz), 7.23–7.26 (1H, t, J = 8.0 Hz), 7.41–7.42 (1H, d, J = 8.0 Hz), 7.47–7.49 (1H, d, J = 8.0 Hz). ^{13}C NMR (125 MHz, $CDCl_3$): δ 37.8, 106.2, 111.5, 121.4, 123.1, 125.1, 127.9, 152.6, 155.4.

4.1.1.5. 2-(Benzofuran-2-yl)ethyl 4-methylbenzenesulfonate (9). Compound (**9**) was prepared following similar procedure as (**8**). After column chromatography on silica gel (100–200 mesh), the pure compound (**9**) was obtained as a colorless solid. Melting point: 69 °C. Yield: 0.46 g, 74%. R_f (4:1, petroleum ether/EtOAc) = 0.49. IR (neat/$CHCl_3$): ν 1599, 1456, 1352, 1172, 979 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 2.27 (3H, s), 3.02 (2H, t, J = 6.0 Hz), 4.27 (2H, t, J = 6.5 Hz), 6.33 (1H, s), 7.06–7.13 (4H, m), 7.14 (1H, d, J = 1.5 Hz), 7.22 (1H, d, J = 8.0 Hz), 7.36 (2H, d, J = 1.0 Hz). ^{13}C NMR (125 MHz, $CDCl_3$): δ 21.6, 28.6, 67.4, 104.3, 110.8, 120.6, 122.7, 123.7, 127.8, 128.5, 129.7, 132.7, 144.8,

153.2, 154.7; HRMS (ESI) $(M+Na)^+$ calculated for $C_{17}H_{16}O_4SNa^+$ = 339.0667, found 339.0665.

4.1.1.6. 3-(Benzofuran-2-yl)propyl 4-methylbenzenesulfonate (10). Compound (**10**) was prepared following similar procedure as (**8**) and was obtained as colorless solid. Melting point: 74 °C. Yield: 70%. R_f (4:1, petroleum ether/EtOAc) = 0.49. IR (neat, $CHCl_3$): ν 1928, 1593, 1450, 1348, 1172, 923, 742, 549 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 1.96–2.05 (2H, m), 2.34 (3H, s), 2.72–2.77 (2H, t, J = 7.2 Hz), 3.99–4.03 (2H, t, J = 6.1 Hz), 6.20 (1H, s), 7.08–7.13 (3H, m), 7.21–7.24 (2H, m), 7.35 (1H, m), 7.68–7.71 (2H, d, J = 7.5 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ 21.6, 24.3, 26.9, 69.2, 102.8, 110.7, 120.3, 122.5, 123.4, 127.9, 128.6, 129.8, 132.9, 144.8, 154.6, 156.9.

4.1.1.7. *endo*-Methyl-2-(benzofuran-2-ylmethyl)-2-azabicyclo[2.2.2]oct-5-ene-7 carboxylate (11a). A suspension of K_2CO_3 (500 mg, 3.16 mmol) in anhydrous CH_3CN (5.0 mL) containing the isoquinuclidine salt **4a** (230 mg, 1.4 mmol) and chloro compound **8** (210 mg, 1.26 mmol) was refluxed for 14 h then cooled to rt and filtered through a pad of Celite, washed with EtOAc (2 × 7 mL). The combined organic extracts were concentrated in vacuo and purified by column chromatography (100–200 mesh silica gel) using EtOAc in petroleum ether (PE) as eluent to obtain the compound **11a** in 71% yield. IR (neat, $CHCl_3$): ν 2949, 1736, 1454, 1194, 1167, 749 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ 1.69–1.72 (1H, m), 1.81–1.86 (1H, t, J = 11.0 Hz), 2.07–2.09 (1H, d, J = 9.5 Hz), 2.61 (1H, br s), 3.05–3.07 (1H, d, J = 10.0 Hz), 3.17–3.19 (1H, br s), 3.56–3.59 (1H, d, J = 14.0 Hz), 3.62 (3H, s), 3.76–3.79 (1H, d, J = 14.5 Hz), 3.89 (1H, br s), 6.20–6.22 (1H, t, J = 5.5 Hz), 6.47–6.50 (1H, t, J = 7.0 Hz), 6.57 (1H, s), 7.18–7.26 (2H, m), 7.47–7.48 (1H, d, J = 7.5 Hz), 7.51–7.53 (1H, d, J = 7.0 Hz). ^{13}C NMR (125 MHz, $CDCl_3$) δ 26.2, 30.9, 44.2, 51.9, 53.7, 54.13, 54.34, 105.1, 111.4, 120.8, 122.7, 123.9, 128.5, 129.4, 135.1, 155.2, 155.8, 174.4. HRMS (ESI) $(M+H)^+$ calculated for $C_{18}H_{19}NO_3H^+$ 298.1443 found 298.1436.

4.1.1.8. *exo*-Methyl-2-(benzofuran-2-ylmethyl)-2-azabicyclo-[2.2.2]oct-5-ene-7-carboxylate (11b). Compound **11b** was synthesized in 64% yield from compounds **8** and **4b**. IR (neat, $CHCl_3$): ν 2943, 1742, 1450, 1193, 1167, 746 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ 1.30–1.35 (1H, t, J = 11.5 Hz), 1.92–1.94 (1H, d, J = 9.0 Hz), 2.10–2.15 (1H, d, J = 12.5 Hz), 2.34–2.36 (1H, d, J = 11.0 Hz), 2.52 (1H, br s), 3.20–3.21 (1H, d, J = 9.0 Hz), 3.40–3.45 (1H, d, J = 14.5 Hz), 3.55–3.57 (4H, m), 3.79 (1H, br s), 6.16–6.19 (1H, t, J = 6.5 Hz), 6.40–6.45 (2H, m), 7.09–7.17 (2H, m), 7.29 (1H, d, J = 8.0 Hz), 7.33–7.36 (1H, d, J = 7.5 Hz). ^{13}C NMR (125 MHz, $CDCl_3$) δ 24.2, 31.1, 45.2, 51.8, 54.02, 54.26, 54.83, 104.4, 110.97, 120.6, 122.5, 123.6, 128.6, 129.7, 135.6, 154.9, 156.4, 174.8. HRMS (ESI) $(M+H)^+$ calculated for $C_{18}H_{19}NO_3H^+$ 298.1443 found 298.1436.

4.1.1.9. *endo*-Methyl 2-(2-(benzofuran-2-yl)ethyl)-2-azabicyclo[2.2.2]oct-5-ene-carboxylate (12a). Compound **12a** was synthesized in 83% yield (326 mg), (R_f = 0.44, PE/EtOAc, 4:1) as a pale yellow oil from **9** and **4a**. IR (neat, $CHCl_3$): ν 3407, 2949, 1732, 1602, 1454, 1251 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ 1.68–1.80 (2H, m), 2.01–2.05 (1H, dt, J = 9.5, 2.5 Hz), 2.59 (1H, br s), 2.63–2.66 (1H, m), 2.86–2.95 (3H, m), 2.97 (1H, dd, J = 9.7, 2.0 Hz), 3.08–3.11 (1H, m), 3.62 (3H, s), 3.82–3.83 (1H, m), 6.18 (1H, dt, J = 6.5 Hz), 6.40 (1H, s), 6.44 (1H, t, J = 7.25 Hz), 7.15–7.21 (2H, m), 7.39 (1H, d, J = 9.0 Hz), 7.45–7.47 (1H, dd, J = 7.0,1.5 Hz). ^{13}C NMR (125 MHz, $CDCl_3$) δ 26.0, 28.1, 30.8, 44.0, 51.8, 54.26, 54.66, 55.97, 56.03, 102.5, 110.77, 110.89, 120.3, 122.49, 122.69, 123.25, 123.66, 128.9, 129.6, 134.8, 154.7, 157.6, 174.4. HRMS (ESI) $(M+H)^+$ calculated for $C_{19}H_{21}NO_3H^+$ 312.1600, found 312.1592.

4.1.1.10. *exo*-Methyl 2-(2-(benzofuran-2-yl)ethyl)-2-azabicyclo[2.2.2]oct-5-ene-7-carboxylate (12b). Title compound (**12b**) was synthesized in 75% yield from **9** and **4b**. IR (neat, $CHCl_3$): ν 2947, 1735, 1454, 1253, 1197, 742 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ 1.36–1.42 (1H, m), 1.91 (1H, dt, J = 9.0, 2.5 Hz), 2.17 (1H, ddd, J = 12.5, 4.3, 2.6 Hz), 2.41–2.45 (1H, m), 2.48–2.52 (1H, m), 2.58 (1H, br s), 2.75–2.99 (3H, m), 3.20 (1H, dd, J = 9.0, 2.0 Hz), 3.55 (3H, s), 3.83–3.85 (1H, m), 6.26 (1H, dd, J = 7.5, 6.5 Hz), 6.40 (1H, s), 6.48 (1H, t, J = 7.5 Hz), 7.14–7.21 (2H, m), 7.36–7.40 (1H, m), 7.45–7.48 (1H, m). ^{13}C NMR (125 MHz, $CDCl_3$) δ 24.4, 27.8, 31.1, 45.4, 51.8, 54.9, 55.2, 55.9, 56.06, 102.51, 102.55, 110.8, 120.31, 120.51, 122.5, 123.14, 123.57, 129.18, 129.94, 135.4, 154.6, 158.1, 174.9. HRMS (ESI) $(M+H)^+$ calculated for $C_{19}H_{21}NO_3H^+$ 312.1600, found 312.1595.

4.1.1.11. *endo*-Methyl 2-(3-(benzofuran-2-yl)propyl)-2-azabicyclo[2.2.2]oct-5-ene-7-carboxylate (13a). Title compound **13a** was prepared in 79% yield from **10** and **4a**. IR (neat, $CHCl_3$): ν 2949, 1735, 1454, 1253, 1197, 1172, 750 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ 1.67–1.78 (2H, m), 1.84–1.95 (3H, m), 2.29–2.34 (1H, m), 2.55–2.61 (2H, m), 2.73–2.83 (2H, m), 2.92 (1H, dd, J = 9.5, 2.0 Hz), 3.07–3.11 (1H, m), 3.63 (3H, s), 3.77 (1H, br s), 6.13–6.16 (1H, m), 6.33 (1H, s), 6.42 (1H, t, J = 7.5 Hz), 7.14–7.22 (2H, m), 7.38 (1H,), 7.45–7.47 (1H, m). ^{13}C NMR (125 MHz, $CDCl_3$) δ 26.16, 26.33, 26.39, 30.9, 43.98 51.8, 54.4, 54.6, 57.2, 102.1, 110.8, 120.3, 122.5, 123.2, 129.11, 129.62, 134.8, 154.8, 159.4, 174.6. HRMS (ESI) $(M+H)^+$ calculated for $C_{20}H_{23}NO_3H^+$ 326.1756, found 326.1751.

4.1.1.12. *exo*-Methyl-2-(3-(benzofuran-2-yl)propyl)-2-azabicyclo[2.2.2]oct-5-ene-7-carboxylate (13b). Title compound was synthesized in 74% yield from **10** and **4b**. IR (neat, $CHCl_3$): ν 2947, 1737, 1600, 1454, 1251, 1197, 750 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ 1.38–1.42 (1H, m), 1.73–1.79 (2H, m), 1.82 (1H, dt, J = 9.0, 2.5 Hz), 2.16–2.21 (2H, m), 2.43–2.56 (3H, m), 2.73–2.79 (2H, m), 3.15 (1H, dd, J = 9.0, 2.0 Hz), 3.73 (3H, s), 3.80 (1H, br s), 6.21 (1H, m), 6.36 (1H, s), 6.44-6.47 (1H, t, J = 7.2 Hz), 7.15–7.21 (2H, m), 7.39–7.41 (1H, d, J = 8.0 Hz), 7.46–7.48 (1H, m). ^{13}C NMR (125 MHz, $CDCl_3$) δ 24.5, 25.8, 26.3, 31.1, 45.5, 51.9, 55.01, 55.14, 56.9, 101.9, 110.8, 120.2, 122.4, 123.1, 129.2, 129.9, 135.3, 154.8, 159.9, 175.1. HRMS (ESI) $(M+H)^+$ calculated for $C_{20}H_{23}NO_3H^+$ 326.1756, found 326.1749.

4.1.1.13. *endo*-Methyl 2-(benzofuran-2-ylmethyl)-2-azabicyclo[2.2.2]octane-7-carboxylate (14). To a stirred solution of compound **11a** (84 mg, 0.28 mmol) in dry methanol (5.0 mL) was added Pd-C (10%) and stirred overnight under hydrogen atmosphere using a hydrogen balloon. Reaction mixture was filtered through a pad of Celite, concentrated in vacuo and purified by silica gel column chromatography (100–200 mesh silica gel) using EtOAc in petroleum ether (PE) as eluent to obtain the compound **14** (79 mg, 93%), (R_f = 0.52, PE/EtOAc, 4:1) as a pale yellow oil. IR (neat, $CHCl_3$): ν 2947, 1736, 1454, 1171, 750 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ 1.53–1.68 (3H, m), 1.77–1.87 (3H, m), 2.03–2.08 (1H, m), 2.63–2.64 (1H, dd, J = 8.0, 1.5 Hz), 2.95–2.96 (1H, dd, J = 8.0, 1.5 Hz), 3.01 (1H, s), 3.02–3.06 (2H, m), 3.66 (3H, s), 3.80–3.88 (3H, m), 6.61 (1H, s), 7.18–7.25 (2H, m), 7.46–7.48 (1H, d, J = 8.0 Hz), 7.52–7.53 (1H, dd, J = 8.0 Hz). ^{13}C NMR (125 MHz, $CDCl_3$) δ 22.3, 24.2, 25.8, 27.2, 39.5, 51.8, 51.9, 52.8, 55.7, 104.9, 111.4, 120.8, 122.7, 123.9, 128.6, 155.2, 155.9, 175.4. HRMS (ESI) $(M+H)^+$ calculated for $C_{18}H_{21}NO_3H^+$ 300.1600 found 300.1604.

4.1.1.14. *endo*-Methyl 2-((1*H*-indol-2-yl)methyl)-2-azbicyclo[2.2.2]oct-5-ene-7-carboxylate (15). A suspension of K_2CO_3 (2.0 g, 14.4 mmol) in anhydrous CH_3CN (10 mL) containing the compound **4a** (1.8 g, 7.2 mmol) and propargyl bromide (80%

in toluene 0.85 g, 7.2 mmol) was refluxed for overnight, then cooled to room temperature, filtered through a Celite pad, and washed with EtOAc (10 mL). The combined organic extracts were concentrated in vacuo and purified by column chromatography on silica gel using EtOAc in petroleum ether as eluent to give alkynylated isoquinuclidine (1.12 g, 76%) as a pale yellow oil (R_f = 0.40, PE/EtOAc, 5:1).

To a mixture of N-Boc-2-iodoaniline (1.4 g, 4.5 mmol), alkynylated isoquinuclidine (1.12 g, 5.45 mmol), $Pd(PPh_3)_2Cl_2$ (5 mol%), and CuI (10 mol%) were added Et_3N (5 mL) and anhydrous benzene (15 mL). The reaction mixture was stirred at room temperature for overnight under argon atmosphere. Tetrabutylammonium fluoride (TBAF) (1.0 M in THF, 6.25 mmol) was added dropwise to the reaction mixture. The reaction mixture was refluxed for 12 h and concentrated in vacuo. The resulting residue was partitioned between water and dichloromethane. The aqueous layer was further extracted with dichloromethane (25 mL). The combined organic extracts were dried over Na_2SO_4 and evaporated in vacuo to obtain the product as pale yellow oil.

The crude mixture was treated with 20% TFA in CH_2Cl_2 (4 mL) at 0 °C and was stirred for 3 h at room temperature. CH_2Cl_2 and other volatiles were removed in vacuo, then saturated aqueous $NaHCO_3$ solution (5 mL) and ethyl acetate (10 mL) were added to the residue. The aqueous phase was extracted with ethyl acetate (2 × 15 mL); the combined organic extracts were washed with brine (10 mL) and the solvent was removed by rotary evaporation. The crude product was purified by column chromatography on silica gel. Elution with 0.5–0.7% MeOH in CH_2Cl_2 gave compound **15** (0.19 g, 12% overall yield from alkynylated isoquinuclidine) as a light pale yellow oil. IR (neat, $CHCl_3$): ν 2951, 1735, 1462, 1175, 747 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ 0.96-0.99 (1H, m), 1.42–1.45 (1H, m), 2.16–2.20 (1H, m), 2.39–2.43 (1H, m), 2.56–2.58 (1H, br s), 3.15–3.23 (1H, dd, J = 9.5, 3.0 Hz), 3.39–3.42 (4H, m), 3.39–3.42 (1H, d, J = 11.5 Hz), 3.60–3.86 (1H, m), 6.26–6.29 (1H, t, J = 7.5 Hz), 6.41 (1H, s), 6.52–6.54 (1H, t, J = 7.5 Hz), 7.21–7.28 (2H, m), 7.43–7.44 (1H, d, J = 6.0 Hz), 7.71–7.72 (1H, d, J = 7.5 Hz), 7.93 (1H, s). ^{13}C NMR (125 MHz, $CDCl_3$) δ 26.4, 34.2, 45.3, 51.3, 51.5, 54.2, 60.0, 86.2, 111.3, 119.0, 121.0, 122.5, 124.3, 128.2, 129.7, 129.8, 135.8, 143.1, 156.7, 175.8. HRMS (ESI) $(M+H)^+$ calculated for $C_{18}H_{20}N_2O_3H^+$ 297.1603 found 297.1607.

4.1.1.15. *endo*-Methyl 2-benzyl-2-azbicyclo[2.2.2]oct-5-ene-7-carboxylate (16). To a round-bottomed flask containing compound **3a** (102 mg, 0.34 mmol) was added 20% HBr–AcOH and stirred for a period of 1 h at room temperature. After the deprotection of CBz group, compound **4a** and benzyl bromide were generated in the reaction mixture and this benzyl bromide was reacted in the next step to get the product **16** according to the following procedure.

The solution was concentrated in vacuo (washing step with petroleum ether was omitted, which was important during conjugation of benzofuran and isoquinuclidine to remove benzyl bromide). To the crude 5.0 mL of anhydrous acetonitrile was added anhydrous K_2CO_3 and heated for 16 h. Filtered through a pad of Celite, concentrated and purified using silica gel column chromatography (100–200 mesh silica gel) using EtOAc in petroleum ether (PE) as eluent to obtain the compound **16** (76 mg, 87%), (R_f = 0.48, petroleum ether/EtOAc, 4:1) as a pale yellow oil. IR (neat, $CHCl_3$): ν 2951, 1734, 1547, 1459, 1175, 749 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ 1.68–1.72 (1H, m), 1.78–1.83 (1H, m), 1.93–1.96 (1H, m), 2.56–2.58 (1H, br s), 2.91–2.93 (1H, dd, J = 9.0, 1.5 Hz), 3.12–3.15 (1H, m), 3.39–3.42 (1H, d, J = 13.0 Hz), 3.61 (3H, s), 3.65–3.68 (1H, d, J = 13.0 Hz), 3.77–3.79 (1H, m), 6.18–6.21 (1H, t, J = 7.0 Hz), 6.46–6.49 (1H, t, J = 7.0 Hz), 7.22–7.25 (1H, m), 7.29–7.34 (4H, m). ^{13}C NMR (125 MHz, $CDCl_3$) δ 26.3, 30.9, 44.3, 51.8, 53.7, 54.5, 61.7, 126.9, 128.3, 128.8, 129.6, 134.9, 139.5, 174.6. HRMS (ESI) $(M+H)^+$ calculated for $C_{16}H_{19}NO_2H^+$ 258.1494 found 258.1501.

4.1.1.16. *endo*-2-(Benzofuran-2-ylmethyl)-*N*-(3 morpholinopropyl)-2-azabicyclo[2.2.2]oct-5-ene-7-carboxamide (21). To a solution of 3-morpholinopropan-1-amine (380 mg, 2.63 mmol) and *endo*-isoquinuclidine carboxylic acid[27] (684 mg, 2.38 mmol) in CH_2Cl_2 (7 mL) was added HOBT (299 mg, 2.2 mmol) followed by EDCI (421 mg, 2.2 mmol). The reaction mixture was stirred at rt for overnight. It was diluted with dichloromethane and washed with water (2 × 10 mL) and brine (10 mL), filtered, dried over Na_2SO_4, and concentrated in vacuo to afford a pale yellow liquid **17**. The compound was dissolved in 20% HBr–AcOH and stirred at room temperature for 1 h. Concentrated in vacuo and the crude product obtained was washed with (2 × 10 mL) petroleum ether and dried to obtain **19** as pale yellow solid.

Compound **19** was dissolved in dry CH_3CN followed by addition of anhydrous K_2CO_3 (907 mg, 6.6 mmol) and the reaction mixture was stirred for overnight in the presence of **8** at 80 °C. Filtered through a pad of Celite and concentrated in vacuo and purified by silica gel column chromatography (100–200 mesh) using EtOAc in petroleum ether (PE) as eluent to obtain the compound **21** (343 mg, 33% overall yield), (R_f = 0.34, petroleum ether/EtOAc, 4:1) as a pale yellow oil. IR (neat, $CHCl_3$): ν 3647, 2957, 1685, 1557, 1194, 789 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 1.73–1.86 (2H, m), 1.81–1.89 (2H, m), 1.94–1.98 (1H, d, J = 9.5 Hz), 2.27–2.36 (5H, m), 2.54–2.56 (2H, m), 2.62–2.71 (2H, m), 2.90–2.93 (1H, d, J = 9.4 Hz), 3.09–3.21 (5H, m), 3.49–3.53 (1H, d, J = 13.6 Hz), 3.74–3.78 (1H, d, J = 13.6 Hz), 3.88 (1H, br s), 6.13–6.17 (1H, t, J = 6.5 Hz), 6.40 (1H, s), 6.42–6.45 (1H, t, J = 7.5 Hz), 7.19–7.30 (2H, m), 7.40–7.46 (1H, d, J = 7.5 Hz), 7.54–7.56 (1H, d, J = 7.2 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ 24.5, 25.9, 26.3, 31.1, 45.6, 51.9, 55.0, 55.1, 56.9, 63.3, 67.1, 101.9, 111.0, 120.3, 122.5, 123.1, 129.2, 129.9, 135.3, 154.8, 159.9, 175.2. HRMS (ESI) $(M+H)^+$ calculated for $C_{24}H_{32}N_3O_3H^+$ 410.2444 found 410.2439.

4.1.1.17. *endo*-2-(Benzofuran-2-ylmethyl)-*N*-(2-methoxyethyl)-2-azabicyclo[2.2.2]oct-5-ene-7-carboxamide (22). To a solution of 2-methoxyethanamine (137 mg, 1.83 mmol) and *endo*-isoquinuclidine carboxylic acid[27] (528 mg, 1.84 mmol) in CH_2Cl_2 (5 mL) was added HOBT (273 mg, 2.01 mmol) followed by EDC (385 mg, 2.01 mmol). The reaction mixture was stirred at rt for overnight. It was diluted with dichloromethane and washed with water (2 × 10 mL) and brine (10 mL), filtered, dried over Na_2SO_4, and concentrated in vacuo to afford a pale yellow liquid **18**. The compound was dissolved in 20% HBr–AcOH and stirred at room temperature for 1 h. Concentrated in vacuo and the crude product obtained was washed with (2 × 10 mL) petroleum ether and dried to obtain **20** as yellow solid.

Compound **20** was dissolved in dry CH_3CN followed by addition of anhydrous K_2CO_3 (635 mg, 4.6 mmol) and the reaction mixture was stirred for overnight in the presence of **8** at 80 °C. Filtered through a pad of Celite and concentrated in vacuo and purified by silica gel column chromatography (100–200 mesh silica gel) using EtOAc in petroleum ether (PE) as eluent to obtain the compound **22** (177 mg, 28% overall yield), (R_f = 0.35, petroleum ether/EtOAc, 4:1) as a pale yellow oil. IR (neat, $CHCl_3$): ν 3659, 2949, 1683, 1550, 1189, 755 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 1.70–1.81 (2H, s), 1.96–2.00 (1H, dd, J = 8.4, 2.6 Hz), 2.29 (1H, br s), 2.59–2.60 (2H, m), 2.96–2.99 (1H, dd, J = 7.0, 2.0 Hz), 3.10–3.14 (1H, m), 3.34 (3H, s), 3.49 (1H, d, J = 13.0 Hz), 3.62 (2H, m), 3.74–3.78 (1H, d, J = 13.0 Hz), 3.87–3.89 (1H, m), 6.18–6.23 (1H, t, J = 7.0 Hz), 6.47–6.49 (1H, t, J = 7.0 Hz), 6.72 (1H, s), 7.23–7.26 (2H, m), 7.45–7.48 (1H, d, J = 7.5 Hz), 7.69–7.72 (1H, d, J = 8.0 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ 26.2, 30.9, 39.4, 44.3, 51.0, 51.7, 53.6, 54.2, 68.9, 110.3, 117.5, 120.4, 122.3, 124.1, 126.9, 128.2, 135.0,

152.8, 155.4, 175.4. HRMS (ESI) $(M+H)^+$ calculated for $C_{20}H_{24}N_2O_3$-H^+ 341.1865 found 341.1867.

4.2. Biology

4.2.1. Materials

DMEM, F12 and FBS were obtained from Gibco-BRL, Life Technologies. Trypsin, soybean trypsin inhibitor, U50488H, Naloxone hydrochloride, Physostigmine, Dynorphin, DAMGO, and anti-mouse IgG-HRP conjugated 2ndary antibody were from Sigma Chemical Co. (USA). Primary antibody of pERK1/2 was purchased from Santacruz (USA). NaI^{125} was from Perkin Elmer (Boston, MA). All other reagents were of analytical grade and obtained locally.

4.2.2. Animals

The experimental procedures involving animals were previously approved by the Institutional Animal Ethics Committee and meet the guidelines of the Government of India. For binding experiments, adult albino Balb/c mice, 25–30 g, were used. Animals were housed four per cage at room temperature and allowed to adapt to laboratory conditions for at least 7 days before the initiation of any experiment. The animals were housed under a standard light dark cycle with free access to food and water, except during testing.

4.2.3. Preparation of ^{125}I-dynorphin, ^{125}I-DAMGO and ^{125}I-DADLE

The opioid peptides, dynorphin, DAMGO and DADLE were labeled with [^{125}I]-sodium iodide as described by earlier study.[43] Briefly, 10 μL of chloramine T (2 μg/μL) dissolved in a 0.2 M phosphate buffer (pH 7.4) to which was added to 10 μL of the above peptides (1 μg/μL) and 1 mCi of $Na^{125}I$. After 40 s, the reaction was terminated by adding 40 μL of sodium metabisulfite (2 μg/μL). Both labeled and unlabeled peptides in the reaction mixture were absorbed in SepPak to free from unreacted $Na^{125}I$. After eluting with a 1 mL solution containing 99.9% acetonitrile with 0.1% TFA, the labeled peptides were separated from the unlabeled peptides by column chromatography on a C_{18} column with elution with a mobile phase of 20:0.2:79.8 acetonitrile/TFA/water. Fractions containing the monoiodinated peptides were pooled and used for subsequent studies.

4.2.4. Receptor binding

4.2.4.1. Membrane preparation. For opioid receptor binding studies, membranes were prepared from mouse brain. Briefly, whole brain was homogenized in ice-cold 50 mM Tris–HCl buffer (pH 7.4), and centrifuged at 20,000*g* for 30 min. The pellet was resuspended in the same buffer, incubated for 20 min at 37 °C, and centrifuged as above. The pellet was resuspended in ice-cold buffer and used for binding assay. Protein concentration was estimated by the method described in earlier study.[44]

4.2.4.2. Radioligand binding. KOR binding was carried out with mouse membrane using [^{125}I]-dynorphin while MOR and DOR binding assay was carried out in mouse membrane using [^{125}I]-DAMGO and [^{125}I]-DADLE, respectively. Briefly, membranes (100-200 μg of protein) were incubated with 2 nM [^{125}I]-dynorphin for 30 min at 37 °C in 50 mM Tris–HCl buffer (pH 7.4). Non-specific binding was determined in the presence of 5 μM concentration of unlabeled U50488H. For MOR and DOR binding, membrane homogenates (300-400 μg protein) were incubated for 2 h at 25 °C in 50 mM Tris–HCl (pH 7.4) containing 2 nM [^{125}I]-DAMGO and [^{125}I]-DADLE respectively in presence and absence of cold naloxone to determine non specific binding. The different test compounds were used at the concentration of 5 μM for studying the displacement of [^{125}I]-dynorphin, [^{125}I]-DAMGO and [^{125}I]-DADLE respectively. The test compounds, which showed significant affinity for KOR and MOR were further evaluated for its IC_{50} value for displacing [^{125}I]-dynorphin and [^{125}I]-DAMGO, using six different concentrations ranging from 1 nM to 25 μM. Following incubation, bound radioligand was collected by filtering under vacuum in a Millipore filtration manifold using glass-fiber filters (GF/B; Whatman, Clifton, NJ), pretreated with 0.5% polyethyleneimine. The filters were washed thrice with ice-cold buffer and the radioactive retained on filters were counted in a liquid scintillation counter (Wallac, model 1049-411, Perkin Elmer, USA).

4.2.5. Screening for agonists

4.2.5.1. Cell culture. C6 glioma cells were used in the study. Cells were grown in DMEM containing 10% fetal calf serum, 50 μg/mL gentamicin, penstrepin 5% CO_2 at 37 °C.

4.2.5.2. ERK assay. ERK phosphorylation, an index of agonistic properties of opioids, was measured by immunoblotting as described previously.[30,31] Following starvation for 24 h, C6 glioma cells were treated as indicated. Antagonists were added to the medium 30 min before stimulation with agonist and compounds. After the indicated stimulation period, medium was removed, and wells of the flasks were washed with ice-cold PBS. Cell lysates were collected in lysing buffer (20 mM HEPES, 10 mM EGTA, 40 mM μ-glycerophosphate, 2.5 mM $MgCl_2$, 2 mM sodium vanadate, 1% NP-40, 1 mM PMSF, 20 μg/mL aprotinin, and 20 μg/mL leupeptin, pH 7.5). Cell lysates were centrifuged at 14,000*g* for 20 min at 4 °C, and protein concentration of the supernatants was determined. Samples were separated by 10% SDS PAGE. Proteins were blotted on PVDF membranes. Ponceau staining of blots was carried out, prior to immunoblotting, to confirm loading of equal amount of proteins in the lanes. Non-specific binding to membrane was blocked with 5% non-fat dry milk in Tris-buffered saline (20 mM Tris, 0.9% NaCl, pH 7.5) for 1 h at 37 °C. The membranes were probed with monoclonal antibody against pERK (1:1000 dilution) for 2–3 h, followed by treating with a peroxidase conjugated secondary antibody.

4.2.6. Evaluation of antinociception by hot-plate test

The method is a modification of those described in earlier studies.[45,46] Balb/c mice (25–30 g) were used for the experiments. The animals were housed under a standard light dark cycle with food and water *ad libitum*.

Mean response time for onset of analgesic response was calculated in three different groups of mice after ip administration of **11a** at three doses, 20, 30 and 40 mg/kg B.W. Mice treated ip with DMSO or 10 mg/kg morphine served as control and positive control respectively. Prior to any treatment, the latency for paw withdrawal was recorded (control latency, CL) for each animal by placing them on a hot plate having a constant temperature of 55 °C. The mean paw withdrawal latency in all the animals before administration of the compound was 7.8 s.

For determining the onset of analgesia which is characterised by loss of nociception, the paw withdrawal latency was measured first at 10 min after treatment. The process was repeated at every 3 min intervals up to 1 h until observation of analgesia in the animals. A cut off latency of 25 s was considered to avoid tissue damage. The time required to achieve the cut off latency in an animal was considered the time of onset of analgesia.

For evaluating percentage maximum possible effect (%MPE), the paw withdrawal latency in the animals were recorded 15 min after administration of test compound or morphine or vehicle and termed as the test latency (TL). The maximum possible effect in percentage (%MPE) was calculated, using the following equation as described[47] with modification:

$\%MPE = 100 \times (TL - CL)/(25 - CL)$.

The mean persistence of duration of analgesic effect was also estimated after the onset of analgesia in three different groups of mice after ip administration of **11a** at three doses, 20, 30 and 40 mg/kg B.W. Mice treated ip with DMSO or 10 mg/kg morphine served as control and positive control respectively.

4.2.7. Tremor test procedures

The tremorigenic activity of the compound **11a** was studied in adult albino Balb/c mice. Doses of physostigmine (0.1, 0.25 and 0.5 mg/kg) were selected based on previous studies[48] to provide a range of tremor. Both the doses of physostigmine and **11a** (20, 30, 40 mg/kg) were administered by ip injection.

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Supplementary data

Supplementary data (synthesis of the compound-**3a**, copies of NMR spectra (^{1}H, ^{13}C) of the new compounds and the tremor test procedure and the results after ip administration of the compound **11a** and physostigmine in mice model) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.09.001.

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