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Letter

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B-973 — A Novel α7 nAChR Ago-PAM: Racemic and Asymmetric Synthesis, Electrophysiological Studies and *in vivo* Evaluation

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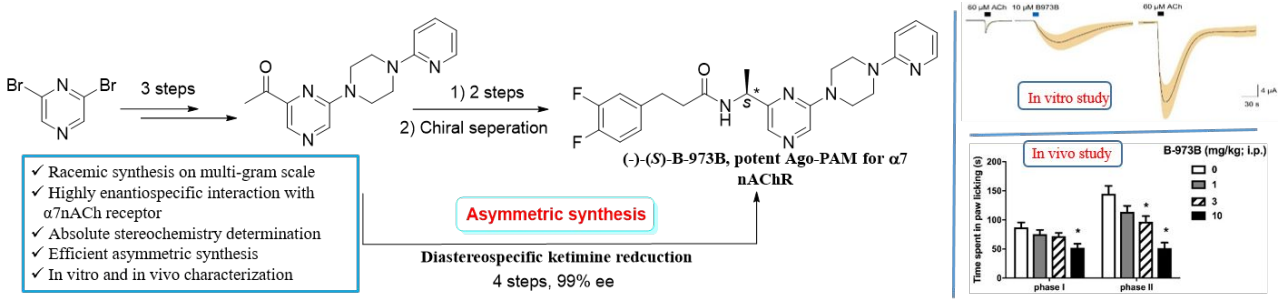


ABSTRACT: We report here the total synthesis of B-973 (5 steps), a recently identified α7 nAChR ago-PAM, its enantiomeric resolution and its electrophysiological characterization in *Xenopus* oocytes to identify (-)-B-973B as the bioactive enantiomer. The asymmetric synthesis of B-973B was accomplished in 99% ee, and X-ray crystallography studies revealed its absolute "*S*" stereochemistry. B-973B was effective in attenuating pain behavior and decreasing paw edema (formalin test), and its analgesic effects were mediated through α7 nAChR. **Keywords:** Alpha7 Nicotinic Acetylcholine Receptors, Ligand-gated Ion Channels, Positive Allosteric Modulators, Ago-PAMs, Neuropathic Pain, Asymmetric Synthesis, Electrophysiological Characterization

Nicotinic acetylcholine receptors (nAChRs) are members of the Cys-loop superfamily of cationic ligand-gated ion channels and are involved in the physiological responses to the neurotransmitter, acetylcholine (ACh).[1-3] These receptors are distributed throughout the central and peripheral nervous systems and play important roles in several (patho)physiological processes. Compounds that selectively target the homopentameric α7 nAChR subtype are being pursued as potential pharmacotherapy agents for treating cognitive dysfunctions in schizophrenia and Alzheimer's disease, as well as for treating neuropathic and inflammatory pain. This type of targeting is safe and is not associated with opioids-like side effects such as addiction liability.[4-7] Attempts to target α7 nAChR has mainly focused on two approaches: partial agonism via the ACh binding site, and positive allosteric modulation (PAM) at sites topographically distinct from ACh binding. Although α7 nAChR-selective and potent (partial) agonists have been developed and studied (pre)clinically, the rapid desensitization of α7 nAChR agents in response to high agonist concentration, together with the possibility of endogenous tone disruption, has led to some concerns regarding their utility as clinical candidates.[2,4,5] PAMs of the α7 nAChR provide an alternative targeting approach that facilitates natural signaling by endogenous ACh and thus is differentiated from partial agonists.[8]

In addition to PAMs, allosteric agonists as well as silent agonists of α7 nAChR have been discovered.[6] However, despite all of these efforts, only limited success has been achieved clinically with galantamine (**Figure 1**). JNJ-39393406 is an α7 PAM that is currently undergoing phase II clinical trials for depressive disorders and smoking cessation.[9,10] Most of the drug candidates that were pursued as α7 nAChR (partial) agonists or PAMs, however, failed in Phase II clinical trials for cognitive disorders due to insufficient efficacy. Recently, a unique class of compounds with dual modes of action — allosteric agonism and positive allosteric modulation —have been discovered and are referred to as "ago-PAMs" (e.g., GAT107, **Figure 1**). [11-14]

Ago-PAMs of α7 nAChR activate receptors through site distinct from orthosteric sites and positively modulate orthosteric cholinergic signaling while preserving the

spatiotemporal features of synaptic transmission.[13,14] Such modulators that act through dual

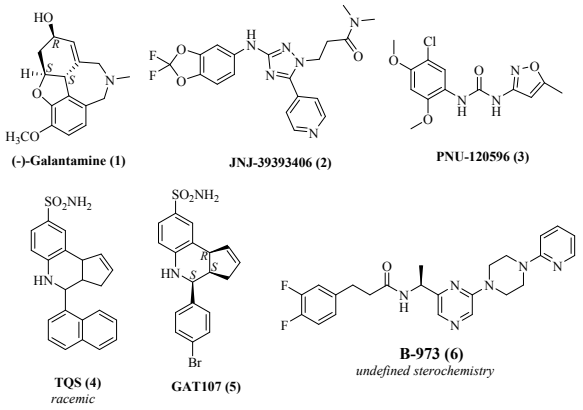
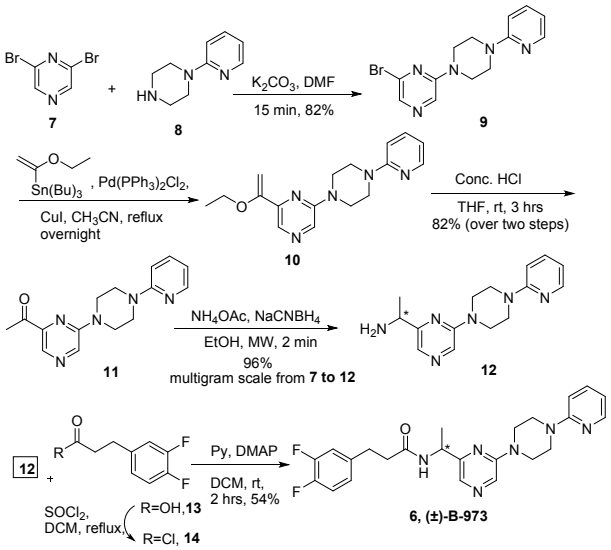


Figure 1: Representative selective positive allosteric modulators of α7 nAChR: an approved drug (**1**), phase II clinical candidate (**2**) and pre-clinical candidates (**3–6**).

modes of action are expected to be more efficacious and potentially address the limitations observed in partial agonists and PAMs in clinical trials. Indeed, we recently demonstrated that one such dual modulator, GAT107, from the tetrahydroquinoline (THQ) class of compounds is an orally bioavailable and efficacious analgesic for treating inflammatory and neuropathic pain in animal models.[15]

Recently, Bristol Myers Squibb reported HTS screening of a diverse small molecule library (>10^6 molecules) using a fluorescence-based assay configured to detect α7 nAChR PAMs.[16] Active compounds identified from the screen included a series of piperazine-containing molecules that displayed robust α7 nAChR PAM activity. One compound from this series, B-973 (**Figure 1**), was unique in that it was shown to be a potent ago-PAM. Although this important work led to the identification of a novel scaffold with α7 nAChR ago-PAM activity, it left several questions unanswered, thus limiting further drug optimization and developmental efforts on this scaffold. Based on the information provided, it is not clear if B-973 is a racemate or a single enantiomer. Neither its absolute stereochemistry nor the activity of the other enantiomer are known. Information on the chemical synthesis of this compound to facilitate *in vivo* studies and library syntheses is also not reported.

Given the therapeutic potential of α7 nAChR ago-PAMs and the excitement surrounding this structurally novel HTS, an effort was made to pursue its synthesis and enantiomeric separation and identify the stereochemical requirements for α7nAChR ago-PAM activity through electrophysiological studies followed by in *vivo* evaluation of the active enantiomer. We report an efficient and scalable synthesis of the racemate as well as of the active enantiomer in 99% ee. Access to copious amounts of the active enantiomer enabled its preclinical evaluation for inflammatory pain (formalin test) in a mouse model, where it was shown to be efficacious. (sentence deleted). The five-step synthetic route reported here was optimized for producing gram-scale quantities of B-973 and is depicted in **Scheme 1**.

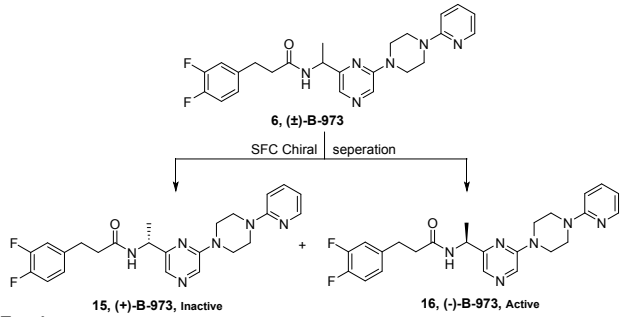


The synthesis begins with the formation of tricyclic bromo pyrazine derivative **9** by mono-substitution of dibromo pyrazine **7** with piperazine **8** in the presence of K_2CO_3 in DMF in 92% yield. To install a keto functionality on **9**, we examined several

Scheme 1. Racemic synthesis of B-973

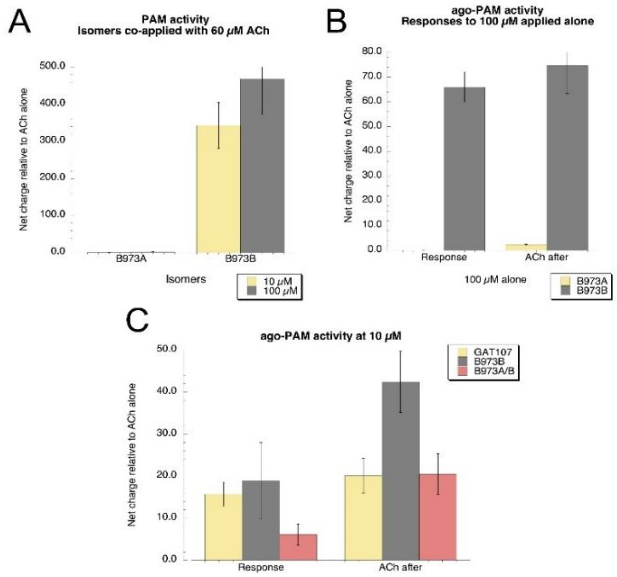
reaction conditions involving BuLi-mediated lithiation followed by quenching with appropriate electrophiles. Our initial efforts were plagued by the formation of a range of undesired products, which was presumably due to the sensitivity of the pyrazine ring to these conditions. We further attempted installing the ketone moiety under mild Pd-catalyzed cross-coupling conditions.[17] Stille cross coupling of compound **9** with tributyl (1-ethoxyvinyl)tin and $Pd(PPh_3)_2Cl_2$ in the presence of CuI yielded the enol ether **10**. The crude product was hydrolyzed with aq. HCl to give the desired ketone compound **11** in 82% yield over two steps. Thus, after obtaining the key keto intermediate **11** in multi-gram quantities, we carried out its conversion to an imine with NH_4OAc, followed by *in situ* reduction with $NaCNBH_4$ to yield racemic amine **12** in 96% yield. The conversion of acid **13** to the corresponding acid chloride **14,** followed by its coupling with the amine fragment **12** in the presence of pyridine and catalytic DMAP yielded the racemic B-973, **6** in 54% yield (**Scheme 1**).

B-973 has one chiral center with two possible configuration. To test whether the allosteric agonist and PAM activity was attributed to a single enantiomer or was present in both compounds, we first developed conditions for the enantiomeric separation of B-973 (**Scheme 2**; see SI). Using chiral super fluid chromatography (SFC), baseline separation of the B-973 enantiomers was accomplished in 99% ee.



Scheme 2. Enantiomeric resolution of racemic B-973.

Both enantiomers were then tested in *Xenopus* oocytes expressing human α7 nAChR with a two-electrode voltage clamp method (Figure 2). Following the measurement of 60 μM ACh control responses, the B-973 isomers were co-applied with a control concentration at 10 or 100 μM. The average net-charge



responses (± SEM) of the oocytes (n ≥ 5) to the A and B isomers, normalized to the initial ACh control responses from the same cells, are shown in **Figure 2A**. While there were negligible effects from the B-973A isomer, the B-973B isomer greatly increased the responses compared to those evoked by ACh alone.

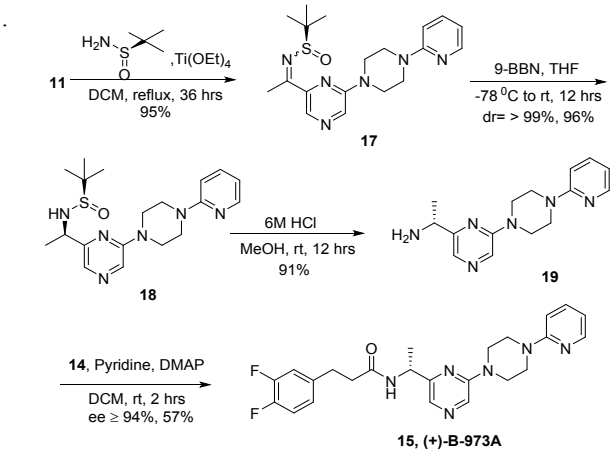
Figure 2: Electrophysiological characterization of the B-973 isomers on human α7 nAChR expressed in *Xenopus* oocytes.

Following measurement of the 60 μM ACh control responses, the B-973 isomers were applied alone at a concentration of 100 μM, followed by another application of 60 μM ACh. The average net-charge responses (± SEM) of the oocytes (n = 6) to the A and B isomers, normalized to the initial ACh control responses from the same cells, are shown in **Figure 2B**. The B-973A isomer failed to evoke a response, and the subsequent ACh controls were not significantly different from the initial controls. In contrast, B-973B evoked large responses, and the ACh responses greatly increased after washout compared to initial controls. The normalized net-charge responses of α7-expressing cells to 10 μM B-973B compared to 10 μM of the previously characterized ago-PAM GAT107 (**Figure 2C**).[12, 13] While the initial allosteric activation produced by the two agents was similar, the primed potentiation of the subsequent 60 μM ACh response was greater ($p < 0.05$) for B-973B than for GAT107. Additionally, the responses to racemic B-973 were smaller ($p < 0.05$) than the response to the B-973B enantiomer. These studies thus concluded that the ago-PAM activity resided exclusively in (-)-B-973B, while (+)-B-973A was inactive at α7 nAChR.

To understand the absolute stereochemical requirements for α7 nAChR ago-PAM activity, we attempted to crystallize both enantiomers to obtain crystals suitable for X-ray studies. However, even after several crystallization attempts, neither of the B-973 enantiomers could be crystallized in an acceptable form. Furthermore, to facilitate structure-activity relationship (SAR) studies, there was a need for easy access to the homochiral version of amine **12** due to the observed high enantio-specificity for α7nAChR activity. We therefore decided to develop an enantioselective approach that would yield the active enantiomer in high ee and allow derivatization of the chiral amine to expand the possibility of obtaining crystals suitable for X-ray studies.

Among the various methods for the preparation of optically active amines[18], we planned to synthesize chiral **12** *via* an approach involving imine formation with chiral *tert*-butanesulfinamide, followed by diastereoselective reduction with the appropriate reducing agent as a key step.[19] Advantageously, both the enantiomers of *tert*-butanesulfinamide are inexpensive and commercially available in high optical purity. Moreover, condensation of *tert*-butanesulfinamide and ketone gives stable ketimine under mild reaction conditions, yields good diastereoselectivity during reduction and allows easy removal of the *N*-*tert*-butanesulfinyl in high yield.

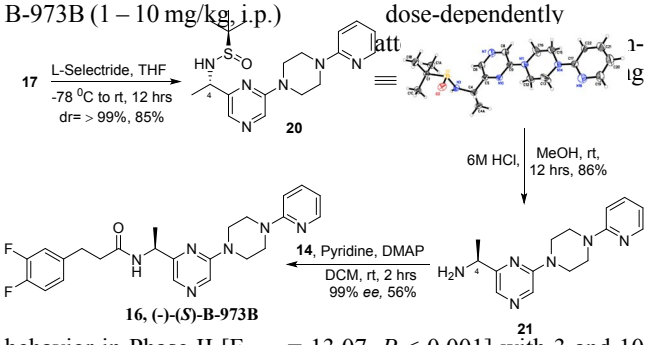
Accordingly, the chiral *N*-*tert*-butanesulfinyl ketimine **17** was synthesized from ketone **11** with *R*-(+)-2-methyl-2-propane sulfonamide in the presence of $Ti(OEt)_4$ in dichloromethane under reflux conditions as a yellow solid in 89% yield. The chiral *N*-*tert*-butanesulfinyl ketimine **17** was stereospecifically reduced by 9-BBN[20-22] in THF at -78 °C to yield sulfinamide **18** as a single diastereomer (dr ≥ 99%; determined by ^1H and ^{13}C NMR spectroscopy) in 96% yield The hydrolysis of the sulfinamide group of **17** was accomplished using 6 M HCl in methanol and gave chiral amine **19** in 86% yield. Finally, the coupling of chiral amine **19** with acid chloride **14** yielded optically pure **15** in 94% ee (**Scheme 3**). By comparing the optical rotation of **15** with the enantiomers obtained from chiral SFC, **15** was identified as the inactive enantiomer.



Scheme 3. Asymmetric synthesis of (+)-B-973A.

To obtain the other enantiomer, *N*-tert-butanesulfinyl ketimine **17** was stereospecifically reduced with L-Selectride[20-22] in anhydrous THF at -78 C to yield **20** as a single diastereomer (dr ≥ 99%; determined by ^{1}H and ^{13}C NMR spectroscopy) in 92% yield. An X-ray crystallographic study revealed the absolute stereochemistry of **20** at C4 to be "*S*". The hydrolysis of the sulfinamide group of **20** using 6 M HCl gave the chiral amine **21** in 86% yield. The absolute "*S*" stereochemistry of the chiral center C4 was further confirmed by a single-crystal X-ray study of *p*-nitrobenzoyl derivative **22** of chiral amine **21** (See SI). Finally, the coupling of **21** with acid chloride **14** yielded the optically pure bioactive isomer **16**, (*S*)-(-)-B-973B, in 56% yield and with 99% optical purity (**Scheme 4**).

We previously demonstrated the antinociceptive and anti-inflammatory efficacy of GAT107 towards pain in a series of mouse models.[15] Here, we tested whether the systemic administration of B-973B would produce antinociception in mouse formalin testing. As seen in **Figure 3A**, B-973B did significantly reduce nociceptive behavior during phase I [$F_{(3,28)} = 3.941$, $P = 0.018$] at the highest dose of 10 mg/kg. Moreover, B-973B (1 – 10 mg/kg, i.p.) dose-dependently attenuated nociceptive behavior in Phase II [$F_{(3,28)} = 13.07$, $P < 0.001$] with 3 and 10 mg/kg doses, differing from the vehicle group.



Scheme 4. Asymmetric synthesis of (-)-B-973B.

In addition, B-973B significantly reduced paw edema [$F_{(3,28)} = 21.05$, $P < 0.001$; Fig 3B] in mice treated with 3 and 10 mg/kg doses, differing from the vehicle group. We next explored the possible role of α7 nAChRs as related to B-973B's effect in the formalin test. To study this, we tested whether the α7 nAChR antagonist, MLA, blocks the antinociceptive effects of B-973B in the formalin test. As shown in Figure 3C, a significant effect of treatment was found [$F_{(3,15)} = 33.05$; $P < 0.0001$]. MLA given alone significantly affected the nociceptive behavior ($P = 0.77$). MLA (10 mg/kg, s.c.) totally blocked the antinociceptive effect of B-973B in both phase I and II of the formalin test ($P < 0.001$).

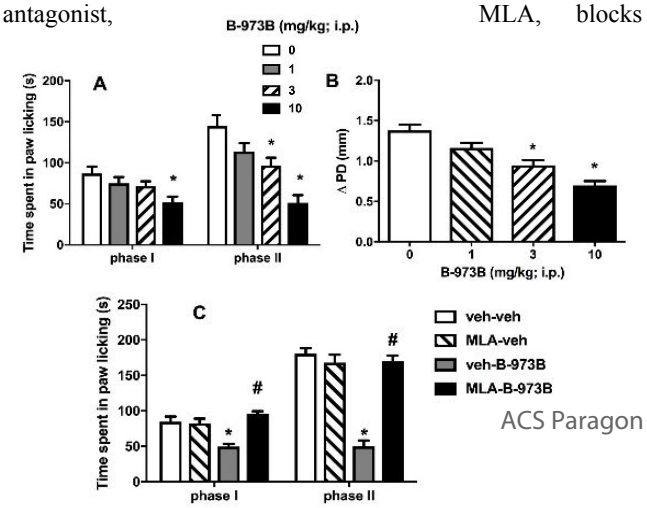


Figure 3: *In vivo* evaluation of the active enantiomer B-973B in a mouse model of inflammatory pain, and establishment of target specificity.

Acute B-973B mainly attenuated pain behavior in the second phase of the formalin test, which is associated with the development of inflammation and spinal dorsal horn sensitization but was only effective in phase I at the highest dose (immediately after formalin injection), which is mediated by C-fiber activity.[23, 24] Moreover, B-973B decreased formalin-induced paw edema, which is consistent with the idea that it acts on the inflammatory phase of the formalin test. Furthermore, using a complementary pharmacological agent (i.e., the α7 nAChR antagonist, MLA), we confirmed that the antinociceptive effects of B-973B in the formalin test were mediated through α7 nAChRs.

Thus, in this work, we have developed the first total, gram-scale synthesis of B-973, a structurally novel α7 nAChR ago-PAM. We also developed conditions for its enantiomeric separation using chiral SFC to give both enantiomers in high enantiomeric excess. The (-)-B-973B enantiomer retained all α7 nAChR ago-PAM activity, while the (+)-B-973B was inactive in electrophysiological assays. Asymmetric synthesis of (-)- B-973B was accomplished in high enantiomeric excess (99% ee). When tested *in vivo*, B-973B was effective in attenuating pain behavior, and decreased formalin-induced paw edema (formalin test). Target specificity of B-973B for analgesic activity was established using MLA. B-973B was as effective as structurally distinct GAT107 in *in vitro* assays and *in vivo* experiments, thus providing a suitable lead for future SAR optimization.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Experimental details, structural characterization data, Chiral SFC separation details, NMR spectra, X-ray study details and biological studies are provided (PDF).

Accession Codes: CCDC 1842480 and 1842483 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

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