DOI: 10.1002/cmdc.200800453

Combinatorial Library Synthesis and Biological Evaluation Pyrazolo[4,3-*e*][1,4]diazepine as a Potential Privileged Structure

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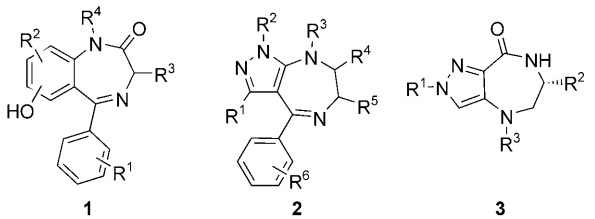
Combinatorial chemistry is increasingly used in the drug discovery process to identify new lead structures. A crucial goal is to find scaffolds that represent classes of molecules capable of binding to multiple receptors with high affinity, termed "privileged structures". The first such structures were the 1,4-benzodiazepin-2-ones **1**, which bind to a broad range of drug targets.[1] Since then, many other privileged structures have been identified.[2–5] Analysis of a prototypical privileged structure, the benzodiazepine scaffold, showed that the conformation effectively mimicked that of β-turns.[6]

Since the discovery of the benzodiazepines, many modified derivatives, displaying a wide pharmacological spectrum, have been developed. Much attention has been paid to the replacement of the fused benzene ring by a heterocyclic ring system.[7–9] Following the discovery of the pyrazolodiazepines **2**, the anxiolytic and central nervous system (CNS) effects of heterocyclodiazepines have been compared with those of benzodiazepines.[9] Recently, Carpino and co-workers disclosed the CB1 cannabinoid receptor antagonism by pyrazolodiazepine-8-one.[10] When diazepines fused to thiophenes, imidazoles, pyrazines, pyrroles and isoxazoles were tested, the general structure–activity relationship (SAR) patterns of benzodiazepinones were most similar to those of the pyrazolodiazepine series.[11] Although some pyrazolodiazepine derivatives have been synthesized,[12–15] the evaluation of diverse structural analogues against different target proteins is lacking. Herein, we report the combinatorial synthesis, biological evaluation and analysis of the pyrazolodiazepine skeleton to determine whether this core structure possesses the properties of a privileged structure,[16] applying the β-turn mimicking concept of tetrahydro-1,4-pyrazolodiazepin-8-one (**3**)[17,18].



1 **2** **3**

We designed an efficient solution phase synthetic route to tetrahydro-1,4-pyrazolodiazepin-8-one (**3**) with three major points of diversity, generating a library of 146 compounds. The idea of positioning appropriate diversity points in the pyrazolodiazepine skeleton was obtained from a computational analysis using a semiempirical calculation[19] for the low-energy conformers of several pyrazolodiazepine structures (Figure 1).

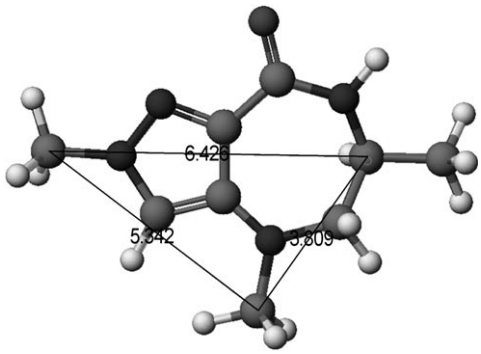
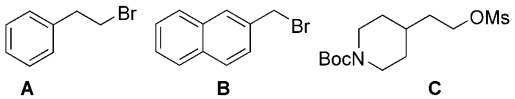


Figure 1. Tetrahydropyrazolo[4,3-*e*][1,4]diazepin-8-one scaffold and distance analysis (in Å).

Computational analysis was performed using the CAChe program (BioMedCAChe Version 5.0, CAChe Scientific, Inc.). The low energy conformer of the pyrazolodiazepine skeleton was obtained by optimizing the geometric calculation in MOPAC using PM3 parameters. Based on an analysis of the ideal distance between Cα atoms of β-turn structures,[18,20] we selected the tetrahydro-1,4-pyrazolodiazepin-8-one (**3**) scaffold, which allows the introduction of substituents at key positions while maintaining the triangular geometries delineated by each pair of Cα atoms among various β-turn types. As shown in Figure 1, the distance between the positions to be substituted can be matched by the ideal distance between Cα atoms of each β-turn moiety.

The method we used to synthesize tetrahydro-1,4-pyrazolodiazepin-8-one (**3**) is depicted in Scheme 1. The carboxylic acid group of commercially available 4-nitro-1*H*-pyrazole-3-carboxylic acid (**4**) was esterified under acid-catalyzed conditions to enable subsequent alkylation reactions. The N-2 position (R^1) of the pyrazole ring was alkylated with three building blocks, which can interact both hydrophobically and ionically. Although two isomers (**6** and **7**) are commonly synthesized



A **B** **C**

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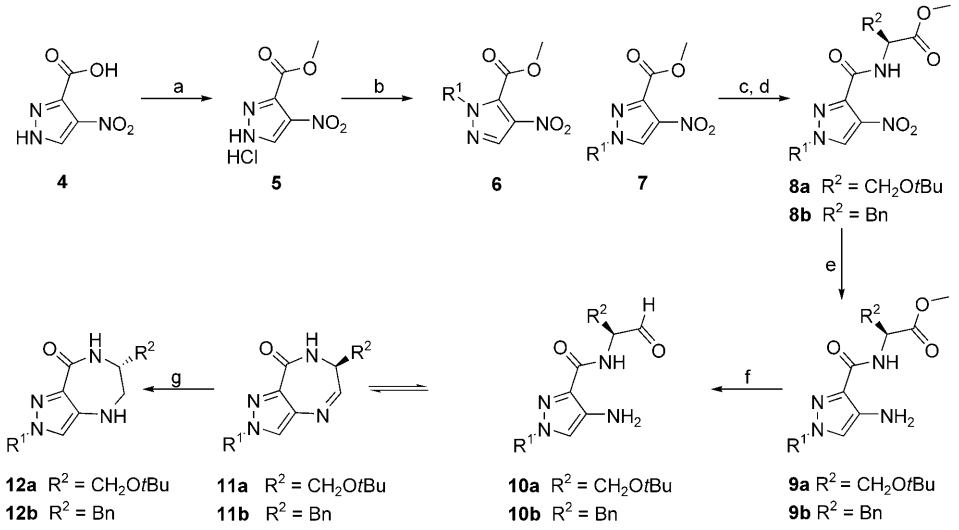
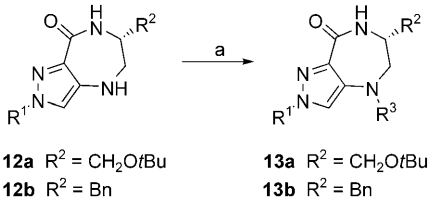
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.200800453.



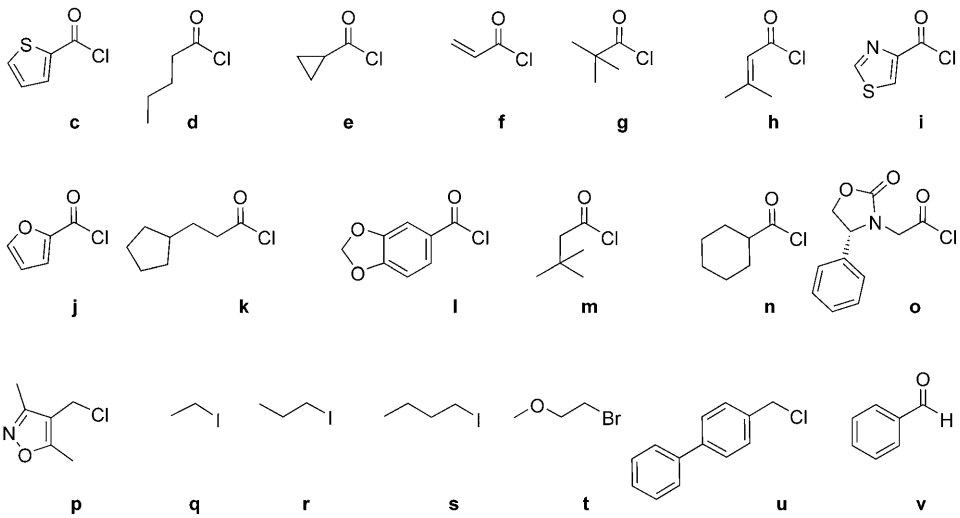
Scheme 1. General synthesis of 6,2-substituted tetrahydropyrazolo[4,3-*e*][1,4]diazepin-8(2*H*)-ones. *Reagents and conditions*: a) CH_3OH, AcCl, 24 h, 95%; b) **A**/**B**/**C**, NaH, DMF, 12 h, 65–84%; c) NaOH in MeOH (1 M), 1 h, >98%; d) L-Ser(*t*Bu)-methyl ester (**a**) or D-Phe-methyl ester (**b**), EDC, HOBt, TEA, CH_2Cl_2, 8 h, 78%; e) Pd/C, H_2, MeOH, 4 h, 98%; f) DIBALH, toluene, 3 h, 60–70%; g) $NaBH(OAc)_3$, AcOH (1%), CH_2Cl_2, 55–60%.

under these conditions, the N-2 regioisomers **7** were predominantly produced in the presence of NaH.[21] The ester group was cleaved using NaOH in MeOH (1 M) to give the free acid, which was then coupled with L-Ser esters and D-Phe esters (compound **8**). The aryl nitro group was reduced by catalytic hydrogenation to give compound **9**. Conversion of the ester to the aldehyde by DIBALH facilitated intramolecular cyclization with the reversible formation of an imine **11**, which was converted to the tetrahydro-1,4-pyrazolodiazepin-8(2*H*)-one scaffold **12** using a standard reducing agent.

The resulting six compounds (**12**) were derivatized at the N-1 position (R^3) of the diazepine ring with a variety of building blocks[22] by parallel solution-phase synthesis to generate a diverse compound library, using an 8-channel parallel synthesizer (Scheme 2). The building blocks were chosen for their hydrophobic, electron donating (alkyl), electron withdrawing (acyl), H bond accepting (heterocycle), and electrophilic (acryloyl) groups including structural motifs seen in MC4 agonists (e.g., phenethyl, piperidine, naphthyl, and benzo[*d*]-[1,3]dioxole-5-carbonyl, etc).[23] These groups were expected to be diverse pharmacophores as well as modifiers of the electronic, steric and lipo/hydrophilic features of the scaffold itself. Generally, small acid chloride building blocks reacted with both the secondary amines and amide NH groups, thus yielding diacylated products.[24] The building blocks with halide functional groups were introduced exclusively at the secondary amines in high yields. A parallel reductive alkylation with aldehyde functionality (group **w**) was also investigated but the reactivity or susceptibility to library synthesis could not surpass those of alkylation using halide and acid chloride. Derivatives containing *tert*-butoxy and Boc groups in the serine side chain (R^2) and a piperidine group (R^1) were deprotected by TFA to yield free OH and NH groups to allow additional interactions with biological targets. After the parallel synthesis of compounds **13** from tetrahydro-1,4-pyrazolodiazepin-8(2*H*)-ones **12** with diversity at the N-1 position of the diazepinone, including five different R^1 and R^2 combinations, purification steps including parallel work up, parallel evaporation by Genvac™ centrifugal evaporator, and parallel chromatography using a Quad3™ purification system gave a library containing 146 final compounds. The purified library compounds



Scheme 2. Solution phase parallel library synthesis. *Reagents and conditions*: a) 1. R^3Cl (**c**–**o**), TEA, CH_2Cl_2, 1 h, 85–90%; 2. R^3X (**p**–**u**), NaH, THF, 8 h, 75–80%; 3. Benzaldehyde (**v**), $NaBH(OAc)_3$, 1% AcOH, CH_2Cl_2, 12 h, 55%.



were randomly characterized by ^{1}H NMR and ESI and MALDI Mass.

To evaluate the pyrazolodiazepine scaffold as a privileged structure these compounds were evaluated against three different drug targets: $P2X_7$ receptor (ion channel), β-secretase (protease), and melanocortin 4 receptor (GPCR). These three targets are representative proteins currently being targeted in drug discovery and development.

The $P2X_7$ receptors, a family of ligand-gated ion channels activated by ATP,[25] are expressed in the periphery of cells of the immune system, such as macrophages and epidermal Langerhans cells.[26] Activation of the ATP-sensitive $P2X_7$ receptor stimulates cation influx[27] and the release of inflammatory cytokines such as interleukin 1β (IL-1β) by macrophages.[28] Thus, this receptor is regarded as a regulator of inflammation, and $P2X_7$ receptor antagonists are actively being investigated as new anti-inflammatory agents, especially for rheumatoid arthritis,[29] inflammatory bowel disease,[30] and chronic obstructive pulmonary disease.[31]

β-Secretase (BACE-1) is a key proteolytic enzyme involved in the N-terminal processing of an integral membrane protein known as amyloid precursor protein (APP) to form amyloid β (Aβ) peptide.[32] The Aβ is aggregated into neuritic plaques, which are found in the brains of patients with Alzheimer's disease (AD).[33] Therefore, inhibitors of BACE-1-mediated APP proteolysis are being developed for the treatment and prevention of AD.

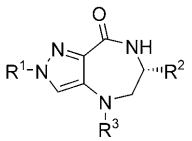
Melanocortin-4 receptor (MC4R), a G-protein-coupled receptor (GPCR) activated by peptide agonists, is a therapeutic target for obesity; targeted disruption of MC4R in mice was found to result in severely obese and hyperphagic animals.[34] Since the determination of the minimal active sequence of the endogenous agonist, melanocyte stimulating hormone (MSH),[35] many SAR and conformational studies have explored the β-turn structural feature of MSH.[36]

Using a cell-based screening system, all library compounds were tested against the three target proteins and the results of the positive compounds are summarized in Table 1. The full results of screening of all compounds are described in the Supporting Information.

Antagonistic activity against human (h) $P2X_7$ receptor was assessed by an ethidium$^+$ accumulation assay[37] using HEK293 cells stably transfected with cDNA encoding the $hP2X_7$ receptor. KN-62 (1-(*N*,*O*-bis(1,5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl)-4-phenylpiperazine),[38] a potent and specific noncompetitive antagonist of the $hP2X_7$ receptor, was used as a positive control. Among the 146 tested derivatives, six compounds had a greater than 50% inhibitory effect at 10 μM against the cytolytic pore formation of $hP2X_7$ receptors induced by 2′,3′-(4-benzoyl-benzoyl)-ATP (BzATP) (Table 1). The antagonistic properties of these six hit compounds enabled an initial analysis of the SAR of a novel series of tetrahydro-1,4-pyrazolodiazepin-8(2*H*)-one compounds against the $hP2X_7$ receptor. Bulky or hydrophobic groups at the R^2 position enhanced antagonistic potency, as exemplified by compounds **13Aa′u**, **13Aau** and **13Cbe** ($CH_2OH < CH_2tOBu < CH_2Ph$). Relatively small groups are better tolerated at the R^3 position, as seen when compounds **13Aau** and **13Aac** (thiophene carbonyl vs 4-phenyl benzyl) and **13Cbe** and **13Cbl** (cyclopropane carbonyl and benzo[*d*][1,3]dioxole-5-carbonyl) are compared. A Boc-piperidine ethyl group at R^1 gave a better IC_{50} value than the phenethyl group (see **13Cbl** and **13Cbe** vs **13Aac**, **13Aao**, **13Aa′u**, and **13Aau**). Furthermore, an energy-minimized conformer of the pyrazolodiazepine skeleton was able to append an appropriate substituent, in a similar fashion to that of KN62, as shown by the superimposition of the two structures (Figure 2).

The pyrazolodiazepine derivatives were also tested for their ability to inhibit BACE-1 as determined by a secreted alkaline phosphatase (SEAP) activity assay using HEK293 cells stably transfected with a mutant form of APP containing alkaline phosphatase and a BACE-1 cleavage site. Compounds **13Bbs**, **13Aau** and **13Ban** displayed >50% inhibitory effect at 10 μM. Interestingly, **13Aau** showed a dual effect on both BACE-1 and $P2X_7R$, which could indicate problematic promiscuity. By employing cell-based assay systems, target protein aggregation

Table 1. Biological evaluation of the pyrazolodiazepine derivatives.



Compound	$R^{1[a]}$	$R^{2[a]}$	$R^{3[a]}$	IC_{50}[b] [μM]		MC4R[c]
				$P2X_7R$	BACE-1	
Positive control (KN62)				0.181 ± 0.074		
13Aac	PE	CH_2OtBu	T-2-C	18.6 ± 2.3		
13Cbl	4-BPE	Bn	BD-5-C	15.7 ± 2.8		
13Cbe	4-BPE	Bn	CPC	4.31 ± 0.5		
13Aao	PE	CH_2OtBu	POAc	18.6 ± 2.6		
13Aa′u	PE	CH_2OH	4-PB	37.9 ± 3.0		
13Aau	PE	CH_2OtBu	4-PB	27.0 ± 5.6	21.5 ± 1.9	
13Bbs	2-MN	Bn	*n*Bu		8.42 ± 0.84	
Positive control (Merck 565788)					0.118 ± 0.001	
13Ban	2-MN	CH_2OtBu	CH-C		52%[d]	2.1
13Bar	2-MN	CH_2OtBu	*n*Pr			1.8
Positive control (NDP-MSH)						10.8

[a] Abbreviations: PE, phenethyl; 4BPE, 4-Boc-piperidine ethyl; 4-PB, 4-phenyl benzyl; T-2-C, thiophene-2-carbonyl; BD-5-C, benzo[*d*][1,3]dioxole-5-carbonyl; CPC, cyclopropane carbonyl; POAc, (*S*)-2-(2-oxo-4-phenyloxazolidin-3-yl)acetyl; CH-C, cyclohexane carbonyl; 2-MN, 2-methyl naphthalene; 2-Mel, 2-methoxy ethyl. [b] IC_{50} = 50% inhibitory concentrations were obtained from concentration-response curves. Data values are expressed as means ± SD. All experiments were repeated at least 2–3 times. [c] Fold increase at 10 μM. [d] % Inhibition at 10 μM.

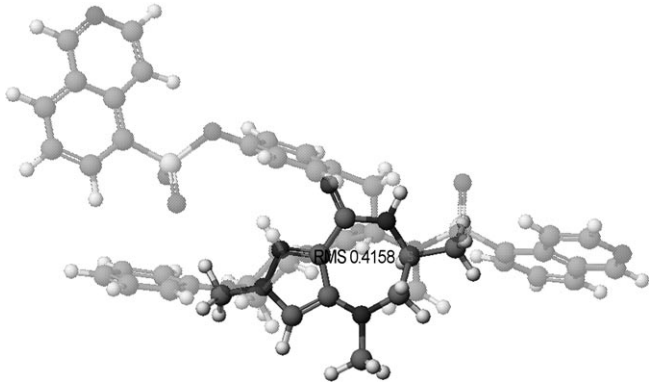


Figure 2. Superimposed structure of the pyrazolodiazepin-8-one skeleton and KN-62

caused by compound promiscuity was avoided. Small changes to the core scaffold of privileged structures can lead to significant activity differences;[39] compound **13 Aa′u**, which has a biphenyl moiety in the same position (R^3) as compound **13 Aau**, displayed 52% inhibition for $P2X_7R$ but only 13% inhibition for BACE-1 (Supporting Information) lending further support to the idea that pyrazolodiazepine is a privileged structure. Among the screened compounds, **13 Bbs** displayed the most potent inhibitory activity (69% at 10 μM) with an IC_{50} value of 8.42 μM upon dose-dependent evaluation ($n=3$). To understand the inhibitory activity of compound **13 Bbs**, a tentative in silico docking study was undertaken. The overlay of the docking structure of compound **13 Bbs** in the complex with inhibitor OM00-3[40] revealed that compound **13 Bbs** can inhibit this enzyme by interacting with each substituent at various subsites (Figure 3). The methyl naphthalene group at R^1 docked into the S1′ and S2′ subsites, whereas the third set of hydrophobic interactions was affected by interaction of the benzyl at R^2 with Leu 30 and Ile 110 in the S3 subsite. The butyl group at R^3 can enhance the inhibitory activity of compound **13 Bbs** by a fourth interaction with the S2 subsite. Furthermore, the activity can be elucidated by binding energy (OM00-3, 0.3 nM, 53.31 kcal mol^{-1}; Merck 565788, 11 nM, 77.27 kcal mol^{-1}; **13 Bbs**, 8 μM, 101.68 kcal mol^{-1}). Over the past decade, most BACE-1 inhibitors have been peptidomimetic and pseudopeptidic compounds, which possess transition state isosteres that interact with two catalytic aspartic acids of the enzyme.[41] However, these inhibitors have limitations, including high molecular weights and multiple H bond donors, limiting their ability to cross the blood–brain barrier and their oral bioavailability. Thus, further optimization of the nonpeptidic pyrazolodiazepines with minimal H bond donor atoms, which interact with the catalytic dyad of the aspartyl protease, would offer a new strategy for the development of BACE-1 inhibitors.

Lastly, the library compounds were examined in a GPCR (MC4R) assay, performed using HEK293T cells transfected with cDNA encoding human MC4R and pCRE-luciferase as previously described.[42] Compounds **13 Bar** and **13 Ban** stimulated MC4R 1.8- and 2.1-fold, respectively, at 10 μM concentration. To understand these results, pyrazolodiazepin-8-one was superimposed onto MT-II,[43] a superpotent cyclic melanotropic peptide (Figure 4). The most important secondary structure of MT-II required for biological activity is the β-turn conformation,[44] which consists of the sequence D-Phe 7–Arg 8–Trp 9. In Figure 4, the Cα positions of the β-turn structure of MT-II were compared with the designed positions of the pyrazolodiazepin-8-one skeleton, as described in Figure 1. We found that the three-point triangular distance of pyrazolodiazepin-8-one (**3**) was well matched with each Cα position. Thus, the results of screening and conformational analysis suggested that the design rationale of tetrahydropyrazolo[4,3-*e*][1,4]diazepin-8(2*H*)-one as a β-turn mimic scaffold is valid. The development of additional small nonpeptidic compounds from the β-turn mimic pyrazolodiazepine, with aryl, basic and hydrophobic groups that mimic the critical sequence Phe–Arg–Trp may yield more potent MC4R agonists.

In conclusion, we have shown here the possibility of pyrazolodiazepine skeleton as a privileged structure. A library of 146

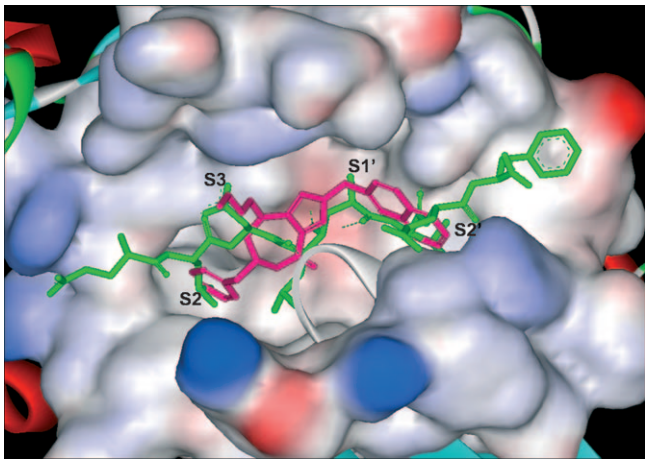


Figure 3. The overlay structure of compound **13 Bbs** (red) with the BACE-1 co-crystal structure with OM00-3 (green). The molecular surface shown represents 10 Å amino acids from compound **13 Bbs** and is colored by electrostatic potential (red, negatively charged; blue, positively charged).

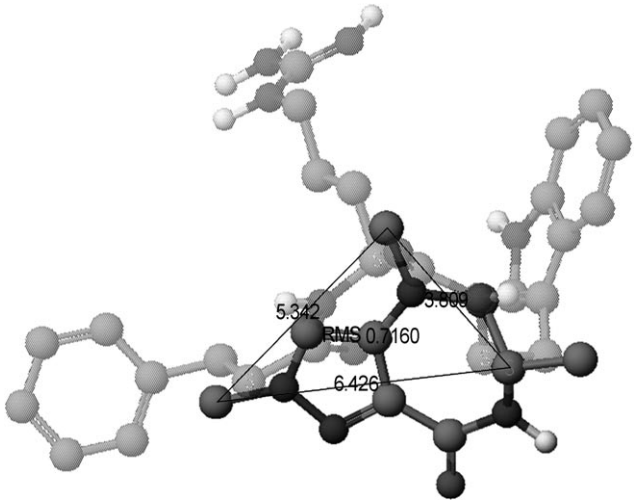


Figure 4. Superimposed structure of the pyrazolodiazepine-8-one skeleton and cyclic Ac-Nle 4–Asp 5–His 6–D-Phe 7–Arg 8–Trp 9–Lys 10-NH_2, MT-II. Only the MT-II sequence D-Phe 7–Arg 8–Trp 9 is shown.

compounds was synthesized from tetrahydro-1,4-pyrazolodiazepin-8(2*H*)-one (**3**) by an efficient solution-phase synthetic route with three major points of diversity. All compounds in the library were assessed for their activity against $P2X_7R$, BACE-1 and MC4R. The privileged nature of the pyrazolodiazepine structure was probed by identifying different hit compounds in screens conducted against each target. The results suggest that the pyrazolodiazepin-8-one skeleton may present appended functionality in biologically relevant topographical shapes and therefore represents a potential privileged scaffold. Compounds based on the scaffold may be used to generate new chemical entities. Further syntheses and optimization of a series of analogues based on the scaffold are presently underway through introduction of crucial pharmacophore moieties.

Acknowledgements

This research was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (grant number 0405-NS01-0704-0001-13) and by a grant of the National R&D Program for Cancer Control, Ministry of Health & Welfare, Republic of Korea (grant number 0720430). We thank Ms. So Deok Lee for performing the ethidium$^+$ accumulation assay, Prof. Yong-Keun Jung in Seoul National University for performing the secreted alkaline phosphatase (SEAP) activity assay, and Dr. Nam Song Choi in ChongKunDang Research Institute for performing the MC4 assay.

Keywords: BACE-1 · MC4R · $P2X_7R$ · privileged structures · pyrazolodiazepines

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Received: December 23, 2008
Revised: January 31, 2009
Published online on ■■ ■■, 2009

COMMUNICATIONS

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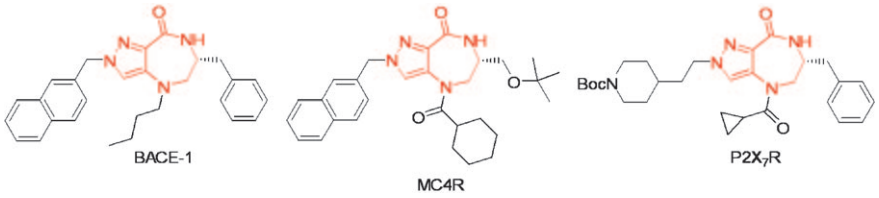
Combinatorial Library Synthesis and Biological Evaluation Pyrazolo[4,3-*e*][1,4]diazepine as a Potential Privileged Structure



A privileged structure: A library of tetrahydro-1,4-pyrazolo-diazepin-8(2*H*)-ones was designed and synthesized to probe the privileged nature of the scaffold. The design strategy included mimicking the three-dimensional conformations of β-turn peptides. Screening against $P2X_7R$, BACE-1, and MC4R gave several hit compounds for each target. The results suggest that pyrazolodiazepin-8-one may represent a potential privileged scaffold.

