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Discovery, synthesis, and structure–activity studies of tetrazole based growth hormone secretagogues

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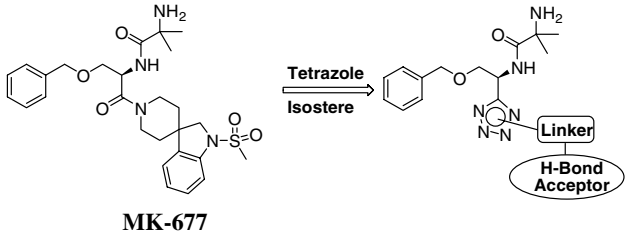
Abstract—A novel class of Growth Hormone Secretagogues (GHS), based on a tetrazole template, has been discovered. In vitro SAR and in vivo potency within this new class of GHS are described. The tetrazole **9q** exhibits good oral bioavailability in rats and dogs as well as efficacy following an oral 10 mg/kg dose in dogs. Solution and solid phase protocols for the synthesis of tetrazole based GHS have been developed.

Growth Hormone Secretagogues (GHS) induce growth hormone (GH) release upon binding to the GHS receptor, a G-protein-coupled receptor located primarily in the pituitary and the hypothalamus. The first GHS reported were the growth hormone releasing peptides (GHRPs), such as the hexapeptide GHRP-6.[1] Subsequently, small, peptidomimetic, orally bioavailable GHS, such as MK-677, were reported[2,3] prior to identification of the natural GHS-receptor ligand, ghrelin in 1999.[4,5]

MK-677[6] (Fig. 1) contains a dipeptide formed by *O*-benzyl-D-serine and 2-methyl alanine. The carboxy-terminus of this dipeptide embedded within MK-677 is connected to a H-bond acceptor through a lipophilic linker (Fig. 1). Published ghrelin structure–activity studies suggest that this dipeptide corresponds to the N-terminus of the GHRPs and ghrelin.[7] This paper describes our design of a novel series of tetrazole based GHS agonists. We maintained the dipeptide fragment present in MK-677 but replaced the amide component with a tetrazole isostere. Disubstituted tetrazoles, which can exist as 1*H* or 2*H* regioisomers, are known bioisosteres for carboxylic acids and their derivatives.[8] In addition to structural novelty, the two possible isomeric cores generated upon replacement of the carboxy group by a tetrazole ring could exhibit different ADME properties from that of MK-677. Although modeling suggested that structural superposition of the 2*H*-tetrazole core with MK-677 was preferred, both the 1*H*- and 2*H*-tetrazole analogs were synthesized and tested in in vitro assays.



MK-677

Figure 1. MK-677 and proposed tetrazole based chemotype.

Our synthesis leading to 1*H*- and 2*H*-tetrazole analogs is depicted in Scheme 1. Coupling of *N*-Boc-*O*-benzyl-D-serine, via its mixed carbonic anhydride,[9] and

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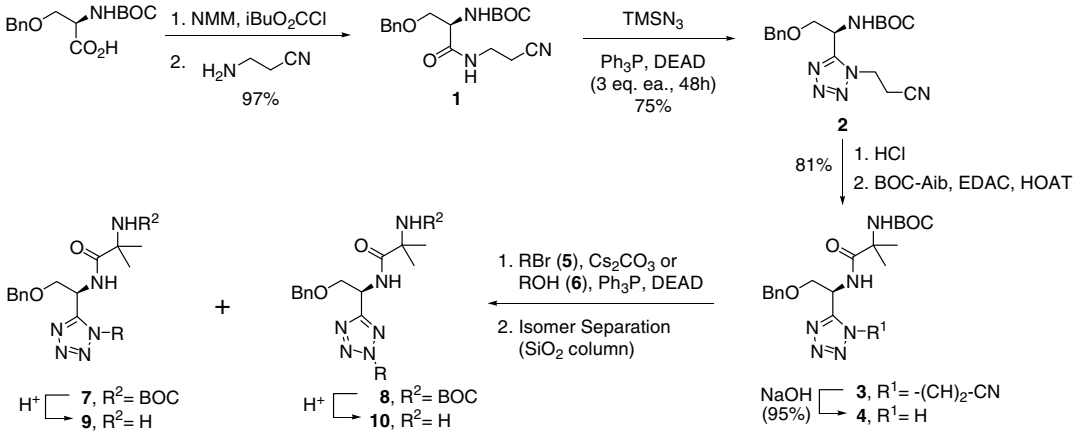
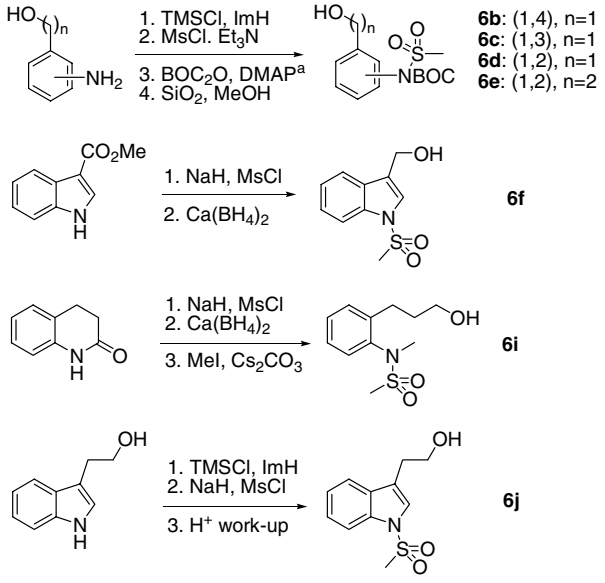


Scheme 1. Synthesis of 1*H*- and 2*H*-tetrazoles.

3-aminopropionitrile furnished the amide **1** in 97% yield. Amide **1** reacted with triphenylphosphine, diethyl azodicarboxylate (DEAD), and azidotrimethylsilane[9,10] to form tetrazole **2** in 75% yield. Boc deprotection followed by amino-acid coupling with *N*-Boc-2-methyl alanine generated amide **3** in 81% yield for the two steps. Removal of the cyanoethyl protecting group yielded the 5-substituted tetrazole **4** in 95% yield. Alkylation of tetrazole **4** with alkyl bromides **5** or via Mitsunobu conditions with alcohols **6** produced a mixture of isomeric 1*H*-/2*H*-tetrazoles **7**/**8** (in >60% yield) ranging in composition from 1:1 to predominantly the 2*H*-tetrazole.[11] Cleavage of the isomeric Boc carbamates **7** or **8** yielded the corresponding amines **9** or **10**, in high yield. Although the linker moiety and/or the H-bond acceptor were occasionally commercially available as alkyl bromides **5**, in most instances this component came from alcohols **6** prepared as depicted in Scheme 2.



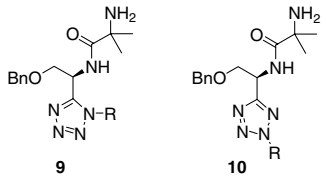
Scheme 2. Synthesis of alcohols **6**. [a]Boc protection prevents interference of the sulfonamide function in the subsequent Mitsunobu reaction. This Boc group is removed along with the Boc group protecting the amine.

We postulated that an alkyl tethered phenyl as the linker joining the tetrazole ring to the methanesulfonamide ($MeSO_2N$) moiety maximized the probability of emulating the putative favorable H-bond acceptor receptor interactions of MK-677 since three different positional isomers are possible for the resulting aryl sulfonamides. Variation of the site of attachment as well as the length and conformational flexibility of the alkyl tether generated tetrazoles **9** and **10**. In vitro characterization revealed that these ligands typically exhibited 5- to 20-fold greater functional activity[12] than binding affinity[13] for the GHS-receptor (Table 1). This fact and the finding that all these tetrazoles are full agonists prompted us to analyze the following SAR based on functional activity.

For the 1*H* series **9a**–**9f**, analogs containing a 1- or 2-carbon linker between the tetrazole and the distal phenyl ring were generally equipotent even if the phenyl ring was incorporated within a polyaromatic system (e.g. indoline **9f**; Table 1). Furthermore, the presence of the $MeSO_2NH$ moiety appeared to confer no advantage (comparison of **9a**–**9b** or **9c**) and could induce a fourfold decrease in potency (**9d**). However, further elongation to a 3-carbon linker generated the phenyl–propyl **9g** and the more constrained phenylpropenyl **9h** analogs, exhibiting, respectively, 5- and 10-fold improved EC_{50} values relative to **9a**. Functional activity of **9i** relative to **9g** was not enhanced upon inclusion of an *ortho* $MeSO_2NMe$ substituent. However, the conformational constraint imposed by linking the two appendages of **9i** to generate the indolyl counterpart **9j** increased potency by fivefold.

In a finding counter to the original molecular modeling prediction, the isomeric 2*H* analogs **10a**–**f** and **10h**–**j** (Table 1) were up to 40-fold less potent agonists than the corresponding 1*H* isomers. Unlike the 1*H* series, tether elongation to a three carbon spacer (**10h**) did not significantly improve potency relative to the parent **10a.** Just as was observed for the 1*H* series, the presence of both the three carbon spacer and the $MeSO_2NMe$ substituent for the 2*H* series enhanced potency the most; however, the SAR response was only about 10-fold (**10i** or **10j** versus **10a**) as compared to 25-fold for the 1*H* series.

Table 1. In vitro activity of tetrazole analogs **9** and **10**



9 **10**

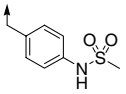
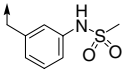
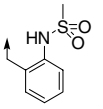
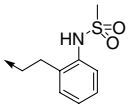
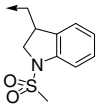
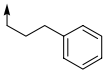
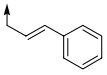
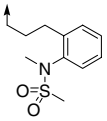
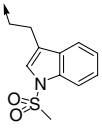
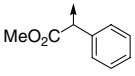
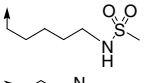
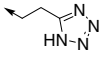
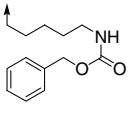
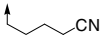
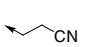
Compound	R	Subst.	K_i[a] (nM)	EC_{50}[b] (nM)
9a		1-*H*	1900	99
10a		2-*H*	4750	1120
9b		1-*H*	>5000	259
10b		2-*H*	>5000	986
9c		1-*H*	>5000	116
10c		2-*H*	>5000	868
9d		1-*H*	4450	390
10d		2-*H*	>5000	426
9e		1-*H*	2200	67
10e		2-*H*	2660	4110
9f		1-*H*	1590	147
10f		2-*H*	>5000	546
9g		1-*H*	200	17
9h		1-*H*	190	12
10h		2-*H*	>5000	517
9i		1-*H*	1720	14
10i		2-*H*	2390	107
9j		1-*H*	970	4
10j		2-*H*	1270	150
9k		1-*H*	470	20
10k		2-*H*	>5000	3170
9l		1-*H*	>5000	75
9m		1-*H*	5000	347
9n		1-*H*	320	10

Table 1 (*continued*)

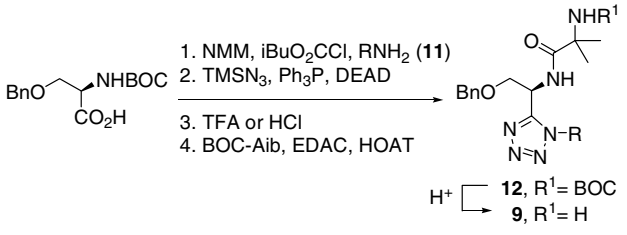
Compound	R	Subst.	K_i[a] (nM)	EC_{50}[b] (nM)
9o		1-*H*	3120	35
10o		2-*H*	>5000	1860
9p		1-*H*	4170	29
10p		2-*H*	>5000	1520
9q		1-*H*	2940	30

[a] See Ref. 13 for detailed description.
[b] See Ref. 12 for detailed description.

To assess the functional activity of an analog containing an H-bond acceptor that, unlike MK-677, has no direct attachment to the phenyl ring, the benzylic carbon of **9a** was substituted with a CO_2Me moiety to generate **9k**. The fivefold improvement in potency of **9k** over **9a** or any of the benzyl sulfonamides **9b**–**9d** indicates that direct attachment of the H-bond acceptor to the phenyl ring is not essential for potency. Moreover, analogs without the phenyl ring (e.g. the pentyl sulfonamide **9l** and tetrazole **9m**) exhibited activities similar to those of benzyl analog **9a**–**9d**. This data demonstrated that either an appropriately tethered phenyl ring (i.e. **9g**) or an H-bond acceptor (**9l**) connected to the tetrazole scaffold afforded significant GHS-receptor activity. The fivefold decrease in potency of **9l** relative to that of **9i** is most likely due to the loss of conformational constraint imposed by the phenyl ring. Increased lipophilicity enhanced in vitro GHS-receptor activity as exemplified by eightfold greater potency of the benzyl carbamate **9n** relative to **9l**.

The emerging SAR pattern favoring disubstituted 1*H*-tetrazoles prompted the development of a regiospecific synthetic route summarized in Scheme 3. Starting with the appropriate primary amine **11**, mixed anhydride coupling with *N*-Boc-*O*-benzyl-D-serine followed by tetrazole formation, Boc deprotection, and aminoacid coupling with *N*-Boc-2-methyl alanine afforded the disubstituted 1*H*-tetrazole **12**. Cleavage of the Boc carbamate **12** provided target amine **9**, as its TFA or HCl salt. The ethyl nitrile **9q**, the phenylpropyl analog **9g**, and the *n*-pentyl Cbz carbamate **9n** were obtained by this last procedure. The methanesulfonamide **12l** was prepared from Cbz carbamate **12n** by hydrogenolysis followed by sulfonylation. Addition of trimethyltin azide[10] to nitrile **12q** generated the bis-tetrazole **12m**.

To ascertain the optimal length of the tether between the tetrazole and an H-bond acceptor, nitriles **9o**–**9q** were evaluated. However, since both the binding affinities

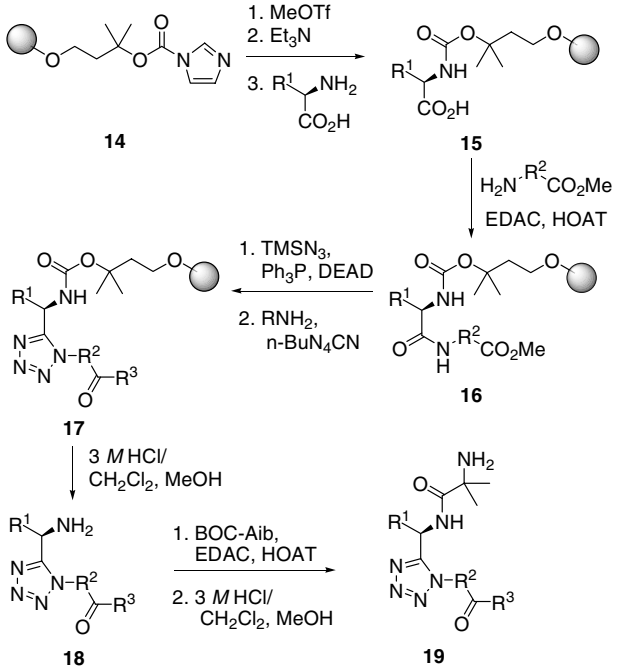


Scheme 3. Synthesis of 1*H*-tetrazoles.

and functional activity are essentially the same for all three compounds, it is apparent that spacing for the nitrile is not relevant unlike the case of the aryl-bearing analogs.

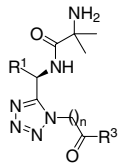
The encouraging in vitro activities exhibited by nitriles **9o**–**9q** prompted further SAR exploration of 1*H*-tetrazoles formed from other aminoacids and/or linked to different H-bond acceptor groups by simple alkyl chains. In order to enable the different permutations between aminoacid residues (R^1), alkyl chains (R^2), and H-bond donor function (COR^3, R^3= OMe or NHR), a solid phase synthesis (Scheme 4) of disubstituted 1*H*- tetrazoles was devised.[14] Anchoring of the D-aminoacid bearing a selected residue (R^1) to a previously activated resin bound (*tert*-alkoxycarbonyl)imidazole **14**[15] generated the corresponding resin bound aminoacid **15**. The alkyl linker (R^2) was introduced by condensation of **15** with an aminoalkyl methylester to yield immobilized **16**. Tetrazole formation,[16] followed by aminolysis (RNH_2) of the methyl ester **16**, afforded the resin bound tetrazole-amide **17** (R^3 = NHR). Acidic cleavage of the resin released amine **18**. Aminoacid coupling of 2-methyl-alalanine with **18** followed by Boc deprotection yielded the target amines **19** as their HCl salts.

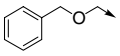
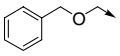
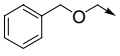
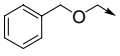
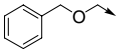
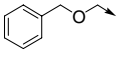
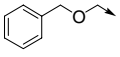
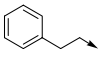
The profile of all the *O*-benzyl-D-serine derived tetrazoles tabulated in Table 2 shows that ester and amide functionality are equivalent replacements for the nitrile as H-bond acceptors. This series differs from that previously discussed as there appears to be little dependency on lipophilicity and spacer length. Comparison of the phenylalkyl amides **19e**–**19g** to the butyl amide **19c** reveals no change in potency as a consequence of increasing lipophilicity. The equivalence in functional and binding activities of esters **19a** and **19b** or the amides **19c** and **19d** reveals the insensitivity to two versus three



Scheme 4. Solid supported synthesis of 1*H*-tetrazoles **19**.

Table 2. In vitro activities of 1*H*-tetrazole-esters and -amides **19a**–**19i**



Compound	R^1	N	R^3	K_i[a]	EC_{50}[b] (nM)
19a		2	–OMe	3380	43
19b		3	–OMe	2380	55
19c		2	–NHnBu	2190	42
19d		3	–NHnBu	1780	30
19e		2	$-NHCH_2Ph$	1070	51
19f		2	$-NH(CH_2)_2Ph$	780	27
19g		2	$-NH(CH_2)_3Ph$	820	59
19h		2	–NHnBu	1370	49
19i		2	–OMe	>5000	200

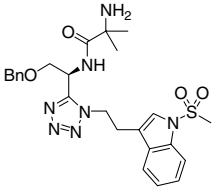
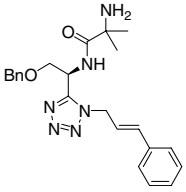
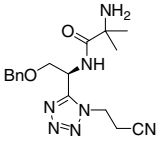
[a] See Ref.13 for detailed description.
[b] See Ref.12 for detailed description.

carbon tethers. The equivalent potencies of the *O*-benzyl serine derivative **19c** and the corresponding homophenylalanine **19h** suggest that the ether moiety does not participate in H-bonding to the receptor. Finally, in this series, the tryptophan-derived **19i** is fourfold less potent than its *O*-benzyl counterpart **19a**. Overall, these tetrazole-esters and -amides have similar in vitro activities to that of tetrazole nitrile **9q**.

The compounds in Table 1 can be divided into three distinct classes of 1*H*-tetrazole agonists: (a) compounds featuring an H-bond acceptor linked to the tetrazole by a lipophilic tether such as **9j**, (b) compounds linked to only a lipophilic group such as **9h**, and (c) compounds with a H-bond acceptor attached to the tetrazole by an alkyl chain such as **9q**. Results from in vivo evaluation of three representative compounds, **9j**, **9h**, and **9q**, in an acute anesthetized IV rat model[17] are summarized in Table 3.

In spite of lower in vitro functional activity, the propionitrile **9q** exhibited superior in vivo activity to that of the

Table 3. In vitro and in vivo activity in the acute anesthetized IV rat model of 1*H*-tetrazoles

Compounds	Structure	EC_{50} (nM)[a]	ED_{50} (μmol/kg)[b]	CLogP
9j		4	>17.4	1.8
9h		12	26.4	2.5
9q		30	8.5	−0.3

[a] See Ref. 12 for detailed description.
[b] See Ref. 17 for detailed description.

other two more liphophilic classes of tetrazoles. This result may be attributed to a potentially better ADME profile of the polar smaller agonist **9q**. The oral bioavailability of **9q** was 56% and 76% in rats and dogs, respectively. When administered orally to beagle dogs at a 10 mg/kg dose, **9q** increased GH mean peak levels to 30 ng/mL compared to 0 ng/mL for vehicle control. Although maximum GH levels occurred between 40 and 45 min post-dose, elevated plasma concentrations of GH persisted for up to 120 min after dosing.

In summary, several novel series of GHS-receptor agonists based on a novel tetrazole template have been described. Reliable solution and solid phase protocols have been developed for the synthesis of the three related sets of analogs **9**, **10**, and **19**. The SAR of the linker and H-bond acceptor were explored. The lead analog **9q** exhibited good oral bioavailability in rats and dogs; moreover, it was orally efficacious in dogs at the 10 mg/kg dose. Further efforts to improve the in vitro and in vivo potency of this 1*H*-tetrazole chemotype will be reported elsewhere.

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11. Isomer separation was usually achieved at this step by column chromatography on silica gel. For a few analogs, 1*H*/2*H* isomeric mixtures were more easily separated by preparative HPLC. ^{1}HNMR easily distinguished between the 1*H* and 2*H* isomers by comparison of the $\Delta\delta$ between the methyls of the 2-aminoisobutyric fragment. The $\Delta\delta$ is significantly larger in the 1*H* isomer than in the 2*H* isomer.
12. H4 glioma cells in which expression of the endogenous human GHS receptor was enhanced by RAGE-activation were utilized for both the functional and binding assays. For details about random Activation for GENE EXPRESION (RAGE)^{TM} technology (Athersys, Inc.), see: Harrinton, J. J.; Sherf, B.; Rundlett, S.; Jackson, P. D.; Perry, R.; Cain, S.; Leventhal, C.; Thorton, M.; Ramachandran, R.; Whittington, J.; Lerner, L.; Costanzo, D.; McElligott, K.; Boozer, S.; Mays, R.; Smith, E.; Veloso, N.; Klika, A.; Hess, J.; Cothren, K.; Lo, K.; Offenbacher, J.; Danzig, J.; Ducar, M. *Nat. Biotechnol.* **2001**, *19*, 440, The EC_{50} was measured by determining intracellular calcium concentration in a FLIPR assay with Ghrelin as having 100% intrinsic functional activity. All EC_{50} values are averages of at least three measurements.
13. Binding affinities were determined by 12-point dose–response curve analysis using membranes from RAGE-based GHSR1a expressing cells. Typically, 0.5 ug membrane was incubated in the presence of 0.02 nM [^{125}I] Ghrelin in 25 mM Hepes (pH 7.2) with 10 mM $MgCl_2$, 2 mM EGTA, and 0.1% BSA in a total volume of 0.2 ml for 1 h in the presence of increasing concentrations of test compound. Reactions were terminated by rapid vacuum filtration over GFB filterplates coated with 0.1% polyethylenimine, and plates were then washed with 0.8 ml of binding buffer and radioactivity subsequently measured by scintillation spectroscopy on a TopCount (Perkin-Elmer) scintillation counter. Curves were fit by nonlinear regression analysis using a four parameter logistic equation, and K_i values were determined using the Cheng-Prusoff equation. Compounds were routinely tested on three separate occasions.
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16. (a) The removal of the large amounts of triphenylphosphine oxide and diethyl hydrazide generated as by-products during the tetrazole formation step is greatly simplified by this solid phase methodology. (b) A portion of the resin bound tetrazole ester was cleaved and coupled to Boc-Aib followed by Boc cleavage in order to evaluate the in vitro activity of the tetrazole ester (**19**, R^3 = OMe).
17. Acute anesthetized rat model: fasted male Wistar rats (200–250 g) were anesthetized via intraperitoneal injection with ketamine (30 mg) and xylazine (10 mg) per kg body weight. Vehicle (10% EtOH/water with 0.9% saline V/V) or the GH secretagogue was administered IV at a volume of 1 mL/kg. After 15 min (the T_{max} for GH in this model), 1.5 mL blood samples were drawn from the abdominal aorta. Plasma samples were then analyzed for rat GH by radioimmunoassay using a modification of the kit supplied by the National Pituitary hormone Center (Dr. A. Parlow, Harbor-UCLA Medical Center, Los Angeles, CA).