Development of a High Specific Activity Sulfur-35-Labeled Sulfonamide Radioligand That Allowed the Identification of a New Growth Hormone Secretagogue Receptor

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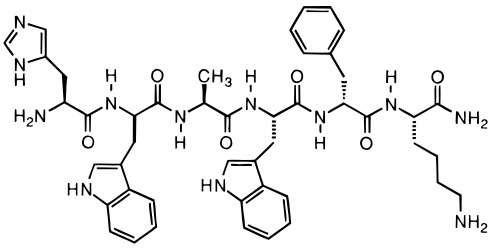
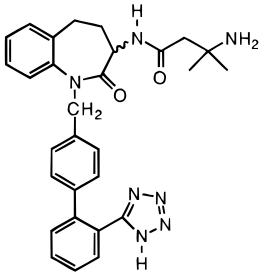
Growth hormone (GH) secretagogues have received considerable attention in the last several years based on promising, ever-widening investigational applications of recombinant growth hormone in animals and in humans. The peptidyl GH secretagogue **1**, GHRP-6 (His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2),[1,2] the benzolactam **2a**, L-692,429,[3,4] and the recently disclosed orally active spiropiperidine-based GH secretagogue **3a** (MK-0677, Figure 1),[5] have been shown to elevate GH in several species, including human. The mechanism(s) of action of these three classes of secretagogues is not clearly understood. Identification of a discrete receptor to which these compounds bind and exert their action has thus far been elusive.

The experimental evidence generated with rat primary pituitary cells shows that the two structurally different secretagogues **3a** and **2a** are mechanistically indistinguishable from the hexapeptide **1** and, hence, are peptidomimetic agonists.[3,6] This is remarkable because small molecule agonists for peptide ligands are rare, presumably due to multiple and precise functional group requirements for agonist activity. We have sought direct evidence to support the contention that **3a** and **2a** are indeed peptidomimetics of the hexapeptide **1**. Competition binding studies with the GH secretagogue receptor would provide strong evidence to show that **1, 2a**, and **3a** are agonist mimetics of one another. These binding studies may require a high specific activity radioligand depending on the receptor abundance and binding affinity. Previous attempts to develop a binding assay using [^{3}H]-[7] and [^{125}I]-labeled[8] peptide ligands derived from GHRP-6 have met with limited success. Generally, the binding was of low affinity and of excessively high capacity. Moreover, the binding affinities did not appear to correlate with the GH secretory activity of the peptides. The lack of correlation of binding affinity and GH secretory activity most likely resulted from the relatively low specific activity (in the case of [^{3}H]GHRP-6) and nonspecific binding properties of the radioligands. Therefore, we explored the synthesis and utility of labeled peptidomimetics such as MK-0677 (**3a**) and its analogs.

In situations where a specific activity of >100 Ci/mmol is required, radioiodine has been the label of

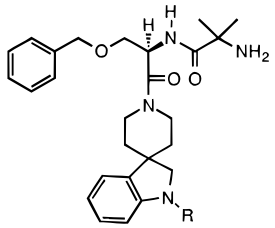


1 GHRP-6(His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2)



2a L-692,429 (R) enantiomer

2b L-692,428 (S) enantiomer



3a R = SO_2CH_3, MK-0677

3b R = SO_2Ph

Figure 1.

choice for the study of receptors.[9] Hence, we initially evaluated the effect of halogen substitution on the phenyl rings of **3a** on GH release from rat pituitary cells. However, incorporation of halogens (e.g. Cl, Br) at the para position of the benzyl and in the 5-position of the spiroindoline phenyl groups lead to an >20-fold loss in intrinsic activity. This indicated that iodine substitution at these positions would not afford a high-potency ligand. In addition, the amino functionality was essential for biological activity, and so conjugation with the widely used Bolton−Hunter reagent[9] did not appear a viable alternative. Therefore, we explored other high specific activity sulfur-35 probes to identify the GH secretagogue receptor.

In this article we detail the synthesis of a high specific activity (700−1100 Ci/mmol) [^{35}S]MK-0677 radioligand.[10] The availability of this [^{35}S]sulfonamide-based radioligand provided the key breakthrough in identifying a saturable, specific, and high-affinity binding site in porcine and rat anterior pituitary membranes which has been linked to the activity of these secretagogues.[6]

We have previously reported that a methanesulfonyl unit on the indoline nitrogen as in **3a** was important in order to obtain good intrinsic activity.[5] Suprisingly, equal activities were obtained using the benzenesulfonyl substitution **3b**. Initially, we chose to explore the preparation of high specific activity benzene[^{35}S]sulfonyl chloride due to the relative ease of synthesis. However, all attempts to sulfonate benzene with carrier free [^{35}S]-sulfuric acid (obtained from NEN at 1300 Ci/mmol) were frustrated by substantial dilution of the specific activity for the resulting benzene[^{35}S]sulfonic acid. This discrepancy was apparently the result of contamination by trace amounts of ubiquitous sulfates in various reagents and/or reaction vessels.

Therefore, we turned our attention to the synthesis of methane[^{35}S]sulfonyl chloride. Much better results were realized using the Strecker reaction[11] to prepare methane[^{35}S]sulfonate from sodium [^{35}S]sulfite. High

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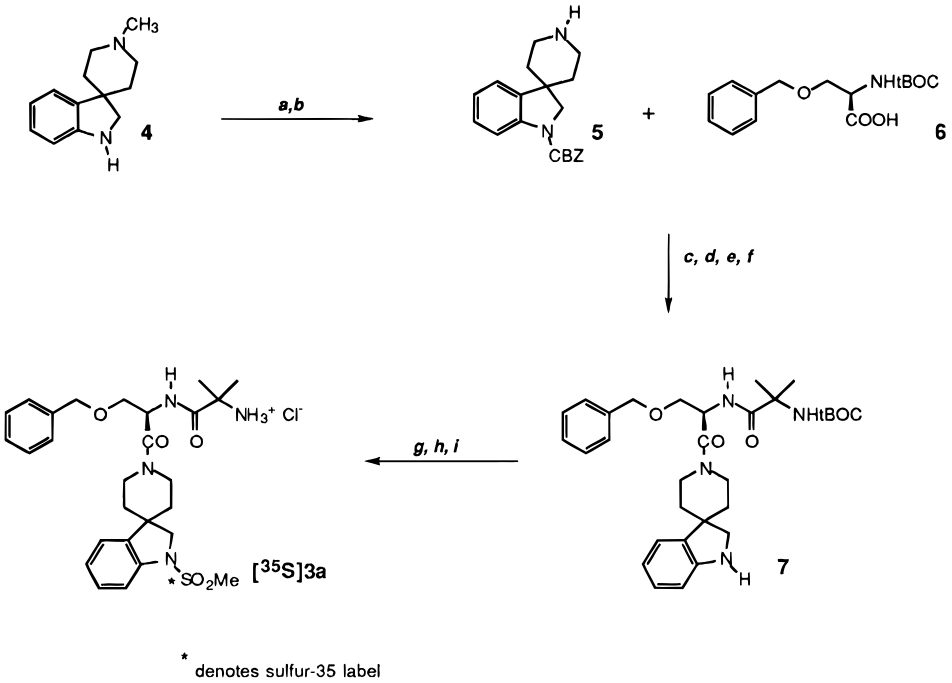
Scheme 1[a]



[a] Reagents: (a) CBZCl, Et_3N, CH_2Cl_2, 0 °C, 30 min; (b) $ClCO_2CHClCH_3$, $ClCH_2CH_2Cl$, reflux, 1 h; MeOH, reflux, 1 h (80%); (c) EDC, HOBT, *N*-methylmorpholine, room temperature, 12 h; (d) TFA; aqueous Na_2CO_3 workup; (e) $HOOCC(CH_3)_2NHtBOC$, EDC, HOBT, CH_2Cl_2, room temperature, 12 h (74%); (f) 10% Pd/C, H_2, EtOH, Et_3N(cat.); (g) Me[^{35}S]SO_2Cl, Et_3N, CH_2Cl_2, room temperature, 30 min; (h) TFA, CH_2Cl_2, room temperature, 30 min; (i) HPLC purification.

specific activity sodium [^{35}S]sulfite could be obtained by reduction of [^{35}S]sulfuric acid on a copper surface with trapping of the generated [^{35}S]sulfur dioxide in sodium hydroxide solution (eq 1). Because the sodium [^{35}S]-sulfite rapidly oxidized to [^{35}S]sulfate, a one-pot reaction system was devised in which excess methyl iodide was added to the sodium hydroxide trap to directly obtain sodium methane[^{35}S]sulfonate (5−10% yield from [^{35}S]-sulfuric acid) along with varying amounts of sodium [^{35}S]sulfate, which was easily recycled (eq 2). Conversion to methane[^{35}S]sulfonyl chloride was best effected (70−75% radiochemical yield) using oxalyl chloride with dimethylformamide in dichloromethane.[12]

$$[^{35}S]H_2SO_4 \xrightarrow[\text{Cu, 200 °C}]{} [^{35}S]SO_2 \xrightarrow[\text{DTT, EDTA}]{\text{Aq. NaOH}} [^{35}S]Na_2SO_3 \quad (1)$$

$$[^{35}S]SO_2 \xrightarrow[\text{Aq. NaOH, MeI, rt}]{\text{Cu, 200 °C}} CH_3[^{35}S]SO_3Na \xrightarrow{(COCl)_2,\ DMF,\ CH_2Cl_2} CH_3[^{35}S]SO_2Cl \quad (2)$$

The synthesis of the necessary amine precursor **7** is illustrated in Scheme 1. Our original four-step synthesis of compounds in the MK-0677 series commenced with an appropriate spiroindoline sulfonamide.[5] For the expedient synthesis of [^{35}S]sulfonamides, we devised a new synthesis of **3a** wherein the sulfonyl unit was incorporated in the penultimate step. Protection of the known spiroindoline **4** as a benzyl carbamate followed by N-demethylation according to the procedure of Olofson[13] gave the spiropiperidine **5** in 80% yield. Standard peptide type coupling of **5** with **6**, removal of the BOC protecting group with strong acid followed by a second coupling with *N*-*t*-BOC-aminoisobutyric acid, and subsequent selective hydrogenolysis of the *N*-CBZ group (Pd/C in ethanol containing a trace of triethylamine) gave **7** in excellent yield.

Optimal conditions for sulfonylation of **7** consisted of adding a maximally concentrated methane[^{35}S]sulfonyl chloride solution to a near saturated solution of **7** in dichloromethane in the presence of triethylamine (Scheme 1). The instantaneous reaction typically produced the desired [^{35}S]sulfonamide in 60−70% radiochemical yields. The remaining radioactivity corresponded to methane[^{35}S]sulfonic acid, formed through hydrolysis by residual water in the presence of amine base. The utilization of more dilute solutions of **7**, even when rigorously dried dichloromethane and triethylamine were used, resulted in almost exclusive formation of the sulfonic acid.[14] Subsequent BOC removal and HPLC purification produced [^{35}S]-**3a** of >99% radiochemical purity.[15]

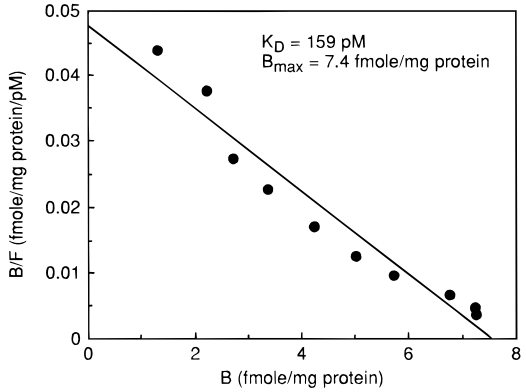


Figure 2. Saturation isotherm of specific [^{35}S]MK-0677 binding in rat anterior pituitary membranes [6] analyzed by Scatchard plot.

Determination of [^{35}S]MK-0677 specific activity by standard UV/concentration measurement proved difficult and unreliable. However, accurate values were readily obtained using μCi amounts of ligand by capillary LC/MS/MS analysis to directly observe the isotopic ratio of ^{32}S/^{35}S. Specific activities ranging from 700 to 1100 Ci/mmol were obtained using different batches of

Table 1. Correlation of Binding Affinities of GH Secretagogues to [^{35}S]MK-0677 Receptor (Swine) and GH Secretory Activity (Rat)

compound	binding affinity (K_i, nM)[a]	EC_{50} secretion (nM)[b]
3a (MK-0677)	0.24	1.3
1 (GHRP-6)	6	10
2a (L-692,429)	63	60
2b (L-692,428)	>5000	>5000

[a] K_i values were determined by using the formula $K_i = IC_{50}/(1+[L]/K_D)$; ligand concentration $L = 100$ nM. [b] Data form rat pituitary cell culture assay[4].

methane[^{35}S]sulfonic acid, presumably a consequence of variable isotopic purity of [^{35}S]sulfate precursor and/or contamination during the preparation of methane-[^{35}S]sulfonic acid.

With high specific activity methane[^{35}S]sulfonamide ligand **3a**, saturable, stereoselective high-affinity binding sites were detected in porcine and rat anterior pituitary membranes.[6] No binding sites were identified in posterior pituitary membranes. Scatchard analysis (Figure 2) indicated the presence of a single class of high-affinity sites with a dissociation constant (K_D) of 161 ± 11 pM which is close to the inhibition constant (K_i) of 240 pM, as determined by displacement of [^{35}S]-**3a** with unlabeled **3a** in competition binding studies. The binding affinities as K_is of the hexapeptide GHRP-6 (**1**) and the structurally diverse small molecules **3a**, **2a**, and **2b** (the inactive stereoisomer) correlated tightly with their GH secretory activities (Table 1). Binding, which required Mg^{2+}, was inhibited by GTP-γ-S, indicating that this newly characterized receptor is G-protein coupled. The GHS receptor was found in exceedingly low concentration (B_{max} = 2.5−7.00 fmol/mg of protein) which underscored the importance of having used the [^{35}S]MK-0677 radioligand **3a** as described in these studies. These results strongly support the designation of **3a** and **2a** as peptidomimetic agonists of GHRP-6.

In conclusion, we have described an efficient synthesis of a high specific activity sulfur-35 labeled sulfonamide for identification of a GH secretagogue receptor. [^{35}S]-MK-0677 was found to possess the necessary combination of high affinity, selectivity, and specific activity required for utilization as a radioligand in the study of this newly discovered receptor. Receptor characterization and use of [^{35}S]MK-0677 may also be of value in the search for the presumed natural ligand of this GH secretagogue receptor. The synthesis of [^{35}S]MK-0677 was accomplished using new methodology to prepare methane[^{35}S]sulfonyl chloride at near theoretical specific activity. This reagent can be coupled to an appropriate amine to provide methane[^{35}S]sulfonamides which possess excellent radiochemical purity and stability.[16] Importantly, this functionality should be considered as an alternative to ^{125}I and may be particularly useful in the synthesis of peptide and non-peptide radiolabeled ligands with long radioactive half-lives and with reduced lipophilicity[17] (π value of $NHSO_2CH_3$ = −1.18[18]) as compared with ^{125}I congeners (π value of I = 1.12[18]). Other interesting and potentially useful observations involving high specific activity sulfur-35 radioligands will be the subject of future publications.

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Supporting Information Available: Experimental procedures and characterization of compounds **5**, **7**, and unlabeled MK-0677; the preparation of methane[^{35}S]sulfonyl chloride; and HPLC radiochromatographs and MS spectra of [^{35}S]MK-0677 (7 pages). Ordering information is given on any current masthead page.

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(15) Preparation of [^{35}S]MK-0677 (**3a**): A solution methane[^{35}S]-sulfonyl chloride (1.5 mCi) in dichloromethane was carefully concentrated via atmospheric distillation in a pear-shaped flask to a minimal volume (~100 μL). This concentrate was added to a mixture of the amine **7** (10 mg, 0.0182 mmol) and triethylamine (5 μL) in 20 μL of dichloromethane at room temperature. The distillation flask was rinsed with dichloromethane (2 × 100 μL), which was added to the amine solution. The mixture was stirred at room temperature for 15 min at which point analysis by HPLC (RX-C8 column, 30% MeCN−0.1% aqueous TFA to

100% MeCN over 30 min, 1 mL/min, radioactivity flow monitor) indicated ~70% *N*-BOC-[^{35}S]MK-0677 (t_R = 20 min) along with ~30% methane[^{35}S]sulfonic acid. The reaction mixture was diluted with 0.5 mL of dichloromethane, washed with saturated sodium bicarbonate solution (2 × 0.5 mL), and dried over sodium sulfate. Measurement of the radioactivity by scintillation counting of the resulting solution indicated 950 μCi (63% yield) to be present with a radiochemical purity of 93% by HPLC analysis (above system). The solution of *N*-BOC-[^{35}S]MK-0677 was concentrated to ~1 mL under a stream of nitrogen at 40 °C, and dimethyl sulfide (100 μL) followed by trifluoroacetic acid (300 μL) was added. The mixture was stirred at room temperature for 1 h at which point analysis by HPLC (above system) indicated complete conversion to [^{35}S]MK-0677 (t_R = 10.5 min). The mixture was concentrated *in vacuo* to near dryness and the resulting residue partitioned between 1% sodium bicarbonate solution (2 mL) and dichloromethane (3 mL). After vigorous stirring, the dichloromethane layer was separated and dried over sodium sulfate. Measurement of the radioactivity by counting the resulting solution indicated 835 μCi (~88% yield) of [^{35}S]-MK-0677 to be present with a radiochemical purity of 92%. Purification was effected by sequential preparative HPLC with attached radioactivity detector using (1) a semiprep RX-C8 column (9.4 mm × 25 cm, 500 μL loop) eluting with a gradient system of 50% MeCN−0.1% aqueous TFA to 70% MeCN over 30 min at a flow rate of 3.5 mL/min (t_R = ~24 min) followed by (2) a analytical PRP-1 column (4.6 mm × 25 cm, 200 μL loop) eluting with a gradient system of 50% MeOH−water with 1 mM of HCl to 90% MeOH−water with 1 mmol HCl over 30 min at a flow rate of 1 mL/min (t_R= ~22 min). The structural identity was established via HPLC coelution with authentic MK-0677. Additional verification was also obtained through analysis of ~20 μCi of the radioligand by capillary LC/MS/MS which also indicated a 10:3.5 ratio of ^{35}S/^{32}S corresponding to a specific activity of 1110 Ci/mmol.

(16) [^{35}S]-**3a** demonstrated remarkable stability (<0.5% loss of radiochemical purity/month) when stored at −20 °C as a solution in 15% methanol−water solution containing a trace of hydrochloric acid.

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