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Radioiodinated (+)-4-[(αR)-α-[(2S, 5R)-4-(Iodopropen-2-yl)-2,5-dimethyl-1-piperazinyl]-3-hydroxybenzyl]-N, N-diethylbenzamide: A Potential Ligand for *In Vitro* and *In Vivo* Investigations of δ-Opioid Receptors

Rikki N. Waterhouse, Michael J. Campa, Jason Park and Edward F. Patz, Jr.
Department of Radiology, Duke University Medical Center, MSRB Box 2610, Research Drive, Durham North Carolina USA 27710.

Summary

(+)-4-[(αR)-α-[(2S, 5R)-4-(Iodopropen-2-yl)-2,5-dimethyl-1-piperazinyl]-3-hydroxybenzyl]-N, N-diethylbenzamide (**1**), a novel radioiodinated derivative of the selective δ-opioid antagonist (+)-BW373U86, was synthesized and evaluated *in vitro* for binding to opioid receptor subtypes. This new compound was found to have high affinity (Ki = 0.57 ± 0.10 nM) and good selectivity for delta (δ) opioid receptors over mu (μ) (Ki μ/δ = 13.6) and kappa (κ) (Ki κ/δ = 175) receptors. The corresponding ^{123}I and ^{125}I derivatives were prepared by oxidative radioiododestannylation from a *trans*-vinyltributyltin precursor. The radiochemical yield was 72-78% EOS (74.3 ± 2.6%, n = 3) for ^{125}I-**1** and 40-62% EOS (53.9 ± 9.8%, n = 3) for ^{123}I-**1**. The specific activities were 200-300 mCi/μmol and >5,000 mCi/μmol for the ^{125}I and ^{123}I-labeled tracers, respectively.

Key Words: delta opioid, receptor, lung cancer, SPECT, imaging

Introduction

Positron emission tomography (PET) imaging with [^{18}F]fluoro-2-deoxy-D-glucose (FDG) has relatively high sensitivity for detecting malignant lesions of the lung which are indeterminate by conventional studies, although its specificity is less than optimal (1, 2). Thus the search continues for a more accurate imaging agent, useful in differentiating benign from malignant lesions and in determining the stage of disease. Due to the accessibility of single

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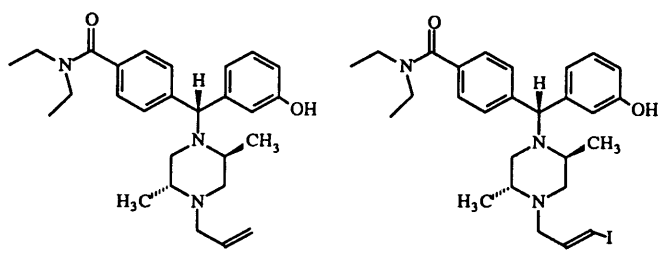
photon emission computed tomography (SPECT), a lung cancer imaging agent labeled with gamma emitting isotopes, such as ^{123}I or ^{99m}Tc, would be invaluable.

In an effort to develop tumor specific imaging agents, we have recently demonstrated the presence of the delta (δ) opioid receptor on small cell lung cancer (SCLC) cells lines and their absence in normal lung (3). We therefore hypothesized that the δ opioid receptor might be an appropriate target for lung cancer imaging.

Currently several radioiodinated peptides targeted against the δ opioid receptor are commercially available for *in vitro* studies, however, they are relatively expensive and are poor candidates for SPECT studies due to rapid *in vivo* metabolism. In addition non-peptidic ligands directed against the same receptor, including radioiodinated analogs of naltrindole (4) and (+)-4-[(αR)-α-[(2S,5R)-4-(allyl)-2,5-dimethyl-1-piperazinyl]-3-[^{123}I]iodo-benzyl]-N,N-diethylbenzamide (5), have also been reported as potential SPECT imaging agents. These are primarily primarily being developed for imaging patients with neurological diseases, in particular, epilepsy, depression and drug addiction, for which changes in brain δ-opioid receptor expression have been demostrated using PET (6-9). To date, there have been no reports describing clinically effective δ-opioid radiopharmaceuticals for SPECT.

To provide δ-opioid-selective radioligands suitable for SPECT imaging applications, we are evaluating new derivatives of (+)BW373U86, a first-generation non-peptidic selective δ opioid receptor ligand (10). (+)BW373U86 binds with high affinity (K_d = 59.3 ± 14.3 pM) to the delta opioid receptor found in SCLC receptors while exhibiting essentially no saturable binding in normal lung (3). The first in this series to be radiolabeled with ^{123}I is (+)-4-[(αR)-α-[(2S,5R)-4-(iodopropen-2-yl)-2,5-dimethyl-1-piperazinyl]-3-hydroxybenzyl]N,N-dieth-ylbenzamide, **1**. Herein is reported the synthesis of **1**, its characterization in opioid receptor binding



(+)BW373U86 **1**

Figure I: Chemical structures of (+)BW373U86 and (+)-4-[(αR)-α-[(2S, 5R)-4-(iodopropen-2-yl)-2,5-dimethyl-1-piperazinyl]-3-hydroxybenzyl]-N, N-diethylbenzamide.

assays, an estimation of its lipophilicity using HPLC methods and the synthesis of the corresponding ^{123}I- and ^{125}I-labeled radiotracers.

Materials and Methods

Proton NMR spectra were recorded on a General Electric QE 300 MHz FT-NMR spectrometer. Chemical shifts were recorded in ppm (δ) in reference to an internal tetramethylsilane standard in deuterochloroform. Coupling constants (J) are reported in Hertz (Hz). High resolution molecular weight determination was performed by electrospray mass spectral analysis (Chemistry Department, Duke University). Flash chromatography was performed using silica gel (Fluka, 70-230 mesh, ASTM) using the solvent systems indicated in the text. For mixed solvent systems, the ratios are given with respect to volumes.

All reagents were purchased from commercial sources and were used without further purification. (+)-4-[(αR)-α-[(2S,5R)-2,5-dimethyl-1-piperazinyl]-3-hydroxybenzyl]-N,N-diethyl-benzamide **2** was synthesized as described and the isolated product exhibited identical chemical and spectral characteristics to those previously reported (12). ^{123}I-Iodide and ^{125}I-Iodide (DuPont Radiopharmaceutical Division, Billerica, MA) were purchased as a solution in 0.1 M sodium hydroxide. HPLC analysis of the radioligand was performed using a Waters 510 HPLC pump, a Waters linear 200 UV detector, and a 925-SCINT AceMate amplifier (EG&G), model NS276 photomultiplier tube base and 905-1 NaI (Tl) crystal (1 x 1", EG&G). For HPLC analyses, a reverse-phase base-deactivated column (Activon, Goldpak Exsil, ODS B, 4.6 x 250 mm (analytical) or 10 x 250 mm (semi-preparative); 10 μm particle size) and the mobile phases used are indicated in the text below.

(+)-4-[(αR)-α-[(2S,5R)-4-Propargyl-2,5-dimethyl-1-piperazinyl]-3-hydroxybenzyl]-N,N-diethyl-benzamide **3**: A portion of the amine **2** (120 mg, 0.3 mmol) was dissolved in dichloromethane (10 ml) and to this was added sodium carbonate (65 mg, 0.6 mmol) and propargyl bromide (40 μl, 0.3 mmol). The resulting mixture was stirred at room temperature for 20 hours and then diluted with water (40 ml). The product was extracted into dichloromethane (3 x 10 ml) and the organic layers were combined, dried over anhydrous magnesium sulfate and the solvent evaporated to provide a crude yellow solid. The product was purified by column chromatography (silica; ethyl acetate; Rf = 0.75) to provide the product as a clear, colorless oil

(131 mg, 84%); ^{1}H-NMR: δ 0.95 (d, 3H, J = 6.0), 1.10-1.42 (m, 9H), 1.83 (t, 1H, J = 10.9), 2.05-2.15 (dd, 2H, J = 10.0, 10.9), 2.5-2.9 (m, 5H), 3.3-3.60 (m, 5H), 5.3 (s, 1H), 2.31 (t, 1H, J = 3) 6.50-6.70 (m, 3H), 7.15 (dd, 1H, J = 8, J = 1), 5.41 (d, 2H, J =), 7.50 (d, 2H, J = 8.11).

(+)-4-[(αR)-α-[(2S,5R)-4-(*Tri-n*-butyltinpropen-2-yl)-2,5-dimethyl-1-piperazinyl-3-hydroxybenzyl]-N,N-diethylbenzamide **4b**: (+)-4-[(αR)-α-[(2S,5R)-4-Propargyl-2,5-dimethyl-1-piperazinyl]-3-hydroxybenzyl]-N,N-diethylbenzamide (100 mg, 0.23 mmol) was dissolved in toluene (3 ml) and to this was added tributyltin hydride (75 μl, 0.27 mmol). The resulting clear, colorless solution was stirred at reflux under a nitrogen atmosphere for 48 hours and then cooled to room temperature. The reaction mixture was loaded directly onto a silica gel column. The product (rf = 0.50) was eluted with ethyl acetate/hexanes (4:1) to provide a clear colorless oil (110 mg, 66%), ^{1}H-NMR: δ 0.75-1.10 (m, 15H), 1.14-1.94 (m, 26H), 2.05-2.15 (dd, 2H, J = 10.0, 10.9), 2.5-2.9 (m, 5H), 3.33-3.60 (m, 5H), 5.30 (s, 1H), 5.98-6.10 (m, 1H), 6.21 (d, 1H, J = 19.8), 6.50-6.70 (m, 3H), 7.22 (dd, 1H, J = 8.1, J = 1.0), 5.41 (d, 2H, J = 8.1), 7.50 (d, 2H, J = 8.1); HRMS (FAB+) 726.4053 (MH^+); calcd for $C_{39}H_{64}N_3O_2Sn$: 726.4056.

Synthesis and purification of (+)-4-[(αR)-α-[(2S,5R)-4-(Iodopropen-2-yl)-2,5-dimethyl-1-piperazinyl]-3-hydroxybenzyl]-N,N-diethylbenzamide **1**: To of dichloromethane (5 ml) was added **4b** (50 mg, 0.78 mmol). To this was added in the absence of light and over a period of 10 minutes a solution of iodine (220 mg, 0.86 mmol) in dichloromethane (5 ml). After stirring at room temperature 15 minutes, the excess iodine was destroyed by the addition of aqueous sodium hydrogen bisulfite (2 ml 0.1 M). Distilled water (25 ml) was then added and the product was extracted into dichloromethane (2 X 25 ml). The organic extracts were combined, dried over magnesium sulfate, and the solvent removed *in vacuo* to provide a crude yellow oil. The product was purified by column chromatography [silica : ethyl acetate, Rf = 0.42] to give a clear, colorless oil (25 mg, 64%): ^{1}H-NMR: δ 0.95 (d, 3H, J = 6.0), 1.10-1.42 (m, 9H), 1.83 (t, 1H, J = 10.9), 2.05-2.15 (dd, 2H, J = 10.0, 10.9), 2.5-2.9 (m, 5H), 3.33-3.60 (m, 5H), 5.55 (s, 1H), 6.26 (d, 1H, J = 14.0), 6.4 (m, 1H), 6.50-6.70 (m, 3H), 7.15 (dd, 1H, J = 8, J = 1), 5.41 (d, 2H, J =), 7.50 (d, 2H, J = 8.11); HRMS (FAB+) 562.1917 (MH^+); calcd for $C_{27}H_{37}N_3O_2I$: 562.1932.

[^{123}I]-(+)-4-[(αR)-α-[(2S,5R)-4-(Iodopropen-2-yl)-2,5-dimethyl-1-piperazinyl]-3-hydroxybenzyl]-N,N-diethylbenzamide ^{123}I-**1**: To a solution of sodium [^{123}I]iodide (17.3 mCi)

in aqueous sodium hydroxide solution (0.1 N, 90 µl) in a 3.0 ml Wheaton vial was added acetic acid (20 µl), chloramine-T (0.5 mg) dissolved in a solution of methanol and water (50 µl, 80:20), followed immediately by a solution **4b** in ethanol (1.2 mg, 200 µl). After standing 1 minute, the reaction mixture was quenched with aqueous sodium metabisulfite (20 µl, 1N) and then made basic by the addition of sodium carbonate (45 mg). The solution was decanted from the solid salts and the product purified by HPLC (mobile phase: methanol/water 80:20; flow rate = 4.0 ml/minute; Rt = 18.4 minutes) to provide 10.7 mCi (62% EOS) of the desired radiotracer. The radiochemical purity of the product was >99% as determined by HPLC analysis. The same procedure was used to prepare ^{125}I-**1**.

The specific activity of the radiotracers were determined by HPLC analysis using a base-deactivated reverse-phase column (Goldpak ODS-B, 4.6 x 250 mm, 10 µm; Rt = 14.6 minutes) and a mobile phase consisting of acetonitrile and water (70:30) with a flow rate of 1.0 ml/min. The specific activity was determined by plotting the mass of **1** injected versus UV detector response at a wavelenth of 254 nm and comparing the UV response obtained when a known quantity of the radiotracer was analyzed.

Lipophilicity estimations: The lipophilicity of **1** was examined by determination of the log $P_{7.5}$ value using a HPLC method previously described (13). Briefly, standard samples having known log P values were analysed using a C18 column (Goldpak Exsil 10mm, 4.6 x 250mm) and a mobile phase of MeOH and phosphate buffer (85:15 v/v, pH = 7.5) at a flow rate of 1.0 ml/min. Relative retention times, RRT (to catechol), were calculated for the HPLC runs of each standard, and a calibration curve of log P vs. log RRT was generated. All sample injections were done in triplicate and the results averaged to provide the final values. A plot of log P versus RRT produced polynomial calibration equations with a correlation coefficient (r^2) of 0.995. The RRT of **1** was then determined and used to calulate its log P value of 3.2.

Isolation of Cell Membranes: Freshly dissected rat brains (minus cerebellum), were dounce homogenized in ice-cold homogenization buffer consisting of 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 µg/ml soybean trypsin inhibitor, and 10 µg/ml each aprotinin and leupeptin. The particulate fraction was pelleted by centrifugation at 40,000 x g for 30 min at 4°C and resuspended in low-Tris homogenization buffer containing 5 mM Tris-HCl (pH 7.5), 1 mM

EDTA, and protease inhibitors as above. Following a 30-min incubation on ice, the suspension was homogenized using an Ultra Turrax tissue disperser (IKA-Works, Inc., Cincinnati, Ohio); unbroken cells and debris were removed by centrifugation at 500 x g for 10 min at 4°C. Membranes remaining in the supernatant fraction were recovered by centrifugation at 40,000 x g as before, resuspended in homogenization buffer, and stored at -70°C until use. Protein content was estimated by a dye-binding assay (BioRad, Richmond, California) using bovine serum albumin as standard.

Binding Studies: The relative binding affinties of experimental compounds for various classes of opioid receptor were investigated by competition binding studies in rat brain membranes (RBM). The radiolabeled ligands used were [^{3}H]naltrindole (20 Ci/mmol), [^{3}H]DAMGO (54 Ci/mmol), and [^{3}H]U-69,593 (44 Ci/mmol) for δ-, μ-, and k-opioid receptors, respectively (14-16). All radioligands were obtained from NEN Life Sciences Product (Boston, MA). All ligands were used at 0.5 nM. RBM (200 μg protein/tube for δ- and μ-opioid receptors, and 500 μg protein/tube for k-opioid receptors) were incubated with the radiolabeled ligand for 1 h at room temperature (22° C) either without competing ligand or in the presence of increasing concentrations of the experimental compound. Incubations were carried out in 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2 mg/ml bovine serum albumin, and protease inhibitors in a total volume of 1 ml. The reaction was terminated by the addition of 4 ml ice cold 50 mM Tris-HCl (pH 7.5) followed immediately by vacuum filtration through Whatman GF/C glass fiber filters (Whatman Paper Ltd., England) using a Brandel model M-48 cell harvester (Brandel Research and Development Laboratories, Gaithersburg, Maryland). The filters were then washed two times with 5 ml ice-cold 50 mM Tris-HCl (pH 7.5). Radioactivity associated with the membranes was quantitated by liquid scintillation spectrophotometry. Specific binding was determined by subtracting the cpm bound in the presence of 10 μM naloxone from that bound in its absence. IC_{50} values were calculated from the displacement curves. Inhibitory constants (K_i) were estimated from the IC_{50} values using the Cheng-Prusoff equation (17). Saturation binding studies were carried out by incubating RBM in the presence of increasing concentrations of [^{125}I]I-BW373. Incubations, filter washes, and radioactivity quantification were carried out as described for competition binding experiments. Non-specific binding was determined using 10 μM naloxone. Dissociation constants (K_d) were calculated from Scatchard plots of the data (11).

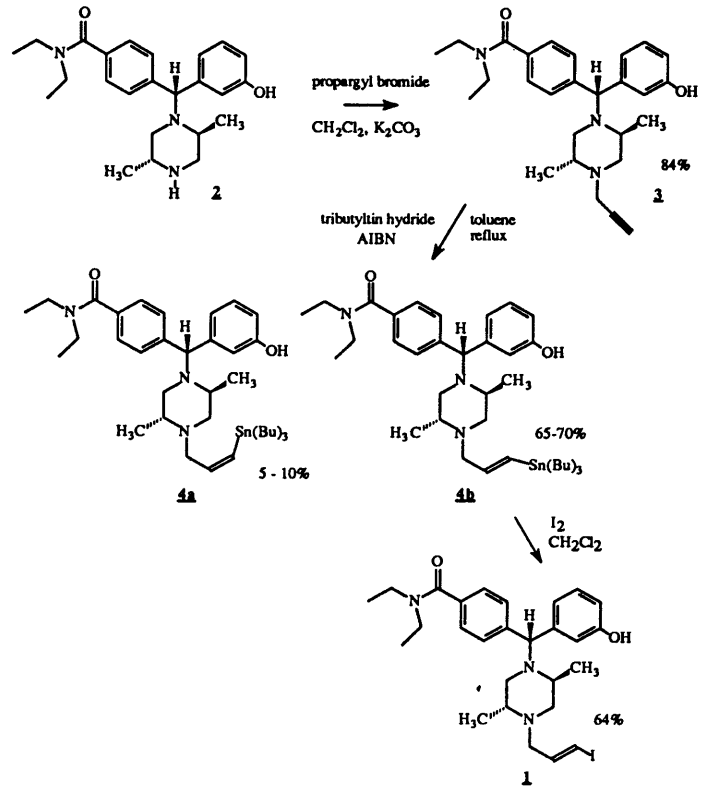
Results and Discussion

The main aim of this study was to develop a radioiodinated δ-opioid receptor radioligand as a potential SPECT imaging agent for the diagnosis of lung cancer. Due to its high affinity and selectivity for δ opioid receptors, we chose to prepare radioiodinated derivatives of the piperazine-based ligand (+)BW373U86. (+)BW373U86 was synthesized in high enantiomeric and diasteriomeric purity using an elegant method described by Bishop and McNutt (12). The enantiomeric purity of the final product was confirmed using HPLC methods with the aid of a chiral cyclodextrin-based column using a mixture of (+/-)BW373U86 as a standard (19). The (+) and (-) enantiomers were readily resolved; the retention times of (+) BW373U86 and the levorotory compound were 24.1 and 31.2 minutes, respectively. From this analysis, the product was found to be >99% enatiomerically pure with the desired (+) isomer being the major component.

To prepare the amine **2**, the allyl group of (+)BW373U86 was cleaved using palladium/charcoal in methanol in the presence of trifluoroacetic acid (3, 19). Alkylation of **2** with propargyl bromide provided **3** in good yield, and **3** was subsequently reacted with an excess of tributyltin hydride to form the *trans*-vinyltributyltin derivative **4b** as the major product (Scheme I). The stereochemistry of **4b** was confirmed by ^{1}H-NMR spectroscopy by determination of the vinylic proton coupling constants (for 4b, J = 19.8 Hz). For vinylstannanes, the coupling constants for vinylic protons in the *cis* configuration are smaller (J = 12-13 Hz) than for protons in the *trans* configuration (J = 18-20 Hz) (20). A small amount (5-10% yield) of the *cis*-isomer was isolated and its structure determined by NMR analysis, however, it was not further characterized.

The target compound **1** was prepared by reacting **4b** with iodine in dichloromethane in the absence of light. The log P of **1** was determined to be 3.2 using HPLC methods, indicating that the compound is moderately lipophilic and should not exhibit a prohibitively high degree of non-specific binding which is characteristic of radioligands having log P values > 4.0 (21).

We have previously demonstrated that compounds possessing log P values between 2.8 and 3.4 determined using this same lipophilicity estimation method can exhibit a high degree of specific binding to their target receptor *in vivo* (13, 22, 23). The characterization of **1** in *in vitro* receptor binding assays revealed high affinity for the δ opioid receptor (Ki = 0.57 ± 0.10 nM)



Scheme I. Synthesis of 1 *Via* a *Trans*-vinyl Tributyltin Precursor.

and, in addition, that the replacement of the allyl group of (+)BW373U86 with a *trans*-iodopropenyl group did not greatly affect the selectivity exhibited by the parent compound for δ over μ and κ opioid receptors (Table I). With this information in hand, we proceeded to synthesize the corresponding radioiodinated derivative of 1.

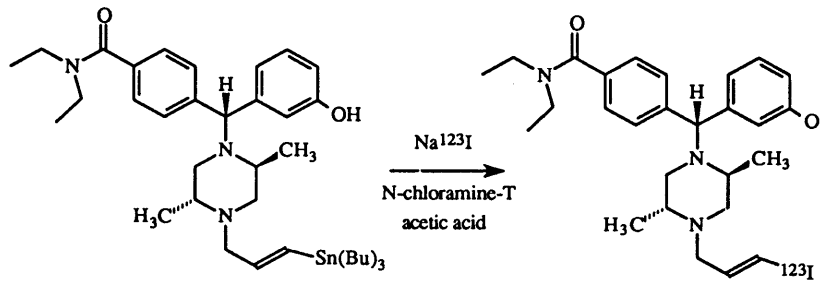
Radioiodination of (+)-4-[(αR)-α-[(2S,5R)-4-((*trans-tri-n*-butyltin)propen-2-yl)-2,5-dimethyl-1-piperazinyl-3-hydroxybenzyl]-N,N-diethylbenzamide, 4b, was accomplished by oxidative electrophilic iododestannylation (Scheme II). [^{123}I]Iodide or [^{125}I]Iodide in aqueous sodium hydroxide solution (0.1 N, 50 μl) was reacted with chloramine-T at acidic pH and to this was immediately added a solution of 4b in ethanol. The reaction mixture was allowed to stand one

minute and was then quenched with aqueous sodium metabisulfite and made basic by the addition of a small quantity of sodium carbonate. The mixture was decanted from the solid salts and the product purified by HPLC to provide ^{123}I-1 in 40-61% yield EOS (Scheme II).

Compound	Receptor Class	Ki (nM)	Kd (nM)
(+)BW373	δ	0.088 ± 0.005	0.13 ± 0.007[a]
1	δ	0.57 ± 0.10	1.45 ± 0.29[b]
1	μ	7.78 ± 0.86[c]	N.D.[d]
1	k	99.56 ± 1.23[c]	N.D.[d]

[a] From (3).
[b] Determined using ^{125}I-1.
[c] Mean ± range of two values determined from two separate experiments; each value calculated from triplicate determinations.
[d] Not determined

Table I. *In Vitro* Binding of 1 to Opioid Receptor Subtypes in Rat Brain Membranes.



4b ^{123}I-1

Scheme II. Synthesis of ^{123}I-1 by oxidative radioiodestannylation.

The radiochemical purity of the product was >99% as determined by HPLC analysis and the radioligand co-eluted with the standard when a spiked aliquot of the purified product was analyzed using identical chromatographic conditions.

To obtain suitable preparations of ^{123}I-1 for use *in vivo*, the appropriate radioactive peak was collected, the mobile phase removed *in vacuo* and the product redissolved in 5% ethanol in saline. The resulting solution was passed through a sterile filter (Millipore, 0.22 mm) into an evacuated sterile vial and diluted with sterile saline to provide a solution that contained approximately 10 mCi of ^{123}I-1 per 10 μl solution. Up to 10.7 mCi of the radiotracer was prepared in this manner.

In conclusion, (+)-4-[(αR)-α-[(2S, 5R)-4-(Iodopropen-2-yl)-2,5-dimethyl-1-piperazinyl]-3-hydroxybenzyl]-N, N-diethylbenzamide, a novel iodinated derivative of the selective δ-opioid antagonist (+)-BW373U86, was synthesized and evaluated *in vitro* for binding to δ, μ and κ opioid receptors. This new compound was found to have high affinity (Ki = 0.57 ± 0.10 nM)

and good selectivity for δ opioid receptors. The corresponding ^{123}I and ^{125}I derivatives were prepared in good yield and high radiochemical purity by oxidative radioiododestannylation of the corresponding *trans*-vinyltributyltin precursor. The total time required for radiosynthesis and purification was approximately 80 minutes. This new radioligand should provide a useful tool for further *in vitro* and *in vivo* studies of δ opioid receptors.

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