Anal. Chem. **2003,** *75,* 6536−6543

Isotope and Affinity Tags in Photoreactive Substance P Analogues To Identify the Covalent Linkage within the NK-1 Receptor by MALDI-TOF Analysis

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Photoreactive analogues of substance P (biotin sulfone−spacer (amino pentanoic or Gly_3)-Arg-Pro-Lys-Pro-(*p*Bzl)-Phe-Gln-Phe-Phe-Gly-Leu-Met(O_2)NH_2) with or without isotope (deuterium) labeling have been synthesized. Deuteriums were present on (d)-biotin or epibiotin sulfone (D_3), on the Gly_3 spacer linker (D_6), or on the Gly in position 9 of SP (D_2). Therefore, peptide analogues could be either unlabeled or tri-, penta-, or hexadeuterated. Results obtained with the use of these peptide analogues show that (d)-biotin sulfone and epibiotin sulfone are not recognized with the same affinity by streptavidin, with (d)-biotin sulfone displaying better affinity for the protein. Photolabeling of the human NK-1 receptor with a 1:1 molar ratio of nondeuterated and deuterated photoreactive substance P (SP) analogues in position 5, followed by combined digestions, purification, and MALDI-TOF mass spectrometry analysis, made the identification of the domain of the receptor covalently linked by the photoreactive SP analogue easier. Indeed, doublets in mass spectra were specific for the covalent complex whereas single peaks could be attributed to contaminating species. This method is particularly suitable when minute amounts of complex have to be analyzed, as in the case of highly hydrophobic G-protein coupled receptors.

Among the different strategies used to elucidate the interaction domain of a ligand within a receptor protein, photolabeling[1,2] is particularly suitable and complementary to mutagenesis studies. After photolabeling, the ligand−receptor complex is subject to one or more enzymatic digestions, chemical cleavage, or both, subsequent purification step(s) being then required to recover the fragments of interest. Besides the design of the photoactivable probes, this purification step is often the cornerstone of such studies. Indeed, HPLC separation requires nanomole amounts of radioactive materials to overcome the adsorption losses. This quantity is a real hindrance to the study of hydrophobic proteins such as recombinant GTP binding-protein coupled receptors that are not easily purified in high amounts from mammal cells. A few years ago, we had developed a procedure involving biotin/streptavidin purification, which does not require the use of a radioactive photoactivable ligand.[3,4] After enzymatic digestions, chemical cleavage, or both, the biotin present at the N-terminus of the photoactivable ligand is used to fish with streptavidin-coated magnetic beads the ligand−receptor complex out of the cellular magma.[4−6] Characterization of the digested complex is then achieved by mass spectrometry, mainly MALDI-TOF because of its high sensitivity (subpicomolar range), its high resolution, and great tolerance to biological media. Recent results using these successive steps have shown the interest and the capabilities of this procedure to define the interaction domain between SP and the human NK-1 (hNK-1) receptor.[4−6]

Despite its apparent versatility and ability, this strategy must however be optimized. Indeed, because of the presence of peaks hard to interpret in the mass spectra, a lot of rather tedious blanks and control experiments are needed before getting final results. The problem is to determine whether these peaks are related to the photoreactive ligand−receptor complex or to contaminating peptides still present after the purification procedure. The fact that the enzyme used can sometimes cleave the receptor and the ligand, and that chymotrypsin-like activity always derives from trypsin during long-time incubations,[7] renders interpretation of the mass spectra even more complicated. Consequently, various enzymatic or chemical digestions and different digestion times are needed to interpret unambiguously the data obtained from mass spectrometry.

We have now developed a strategy complementary to the procedure we used so far. This method, derived from the isotope-coded affinity tag method in proteomic studies,[8,9] is based on the isotopic (deuterium) labeling of the photoactivable ligand, to get

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10.1021/ac034512i CCC: $25.00
Published on Web 10/24/2003

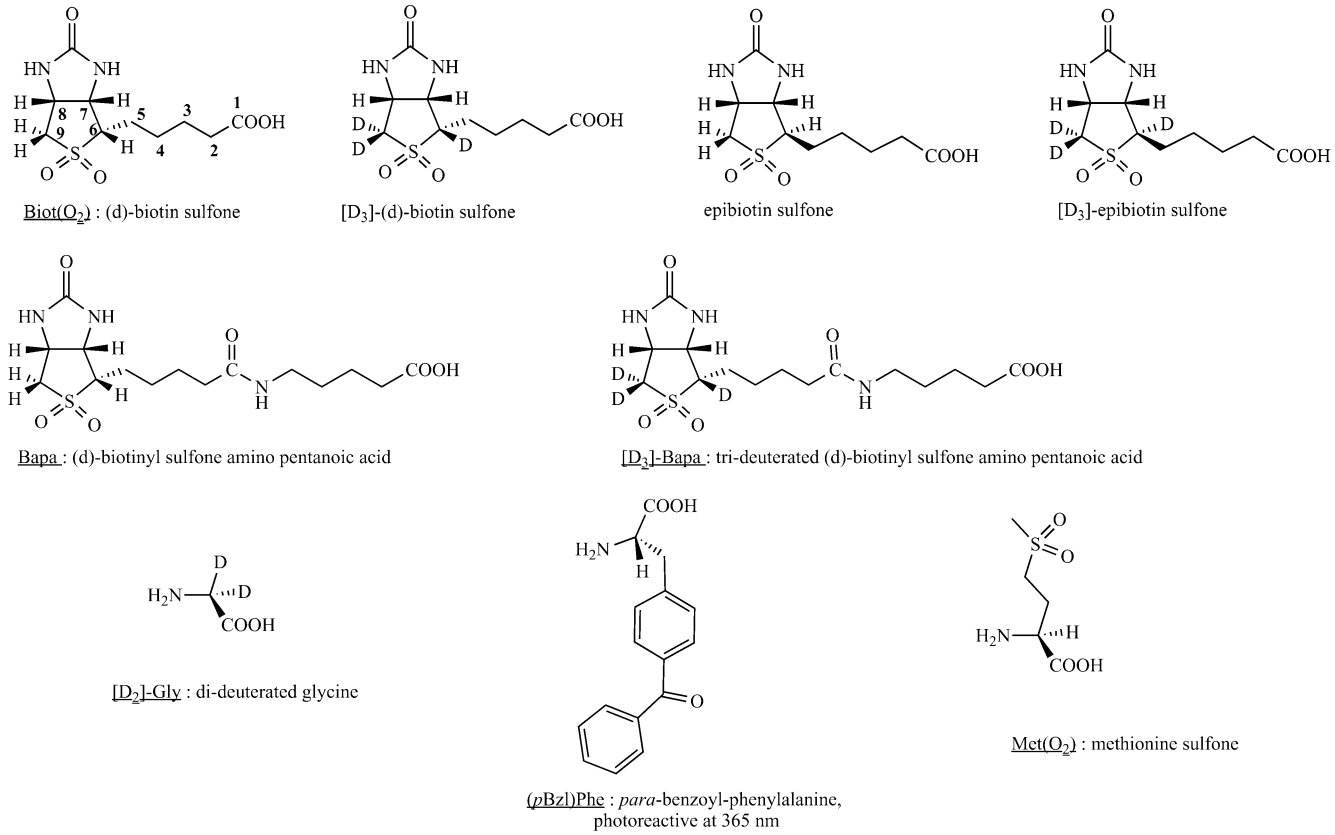

Figure 1. Schematic representation of the chemical modifications incorporated into the sequence of SP as described in the text.

a direct unambiguous reading of the relevant peaks related to the ligand−receptor covalent complex. The photolabeling experiments are carried out with a 1:1 ratio of deuterated and nondeuterated photoactivable nonradioactive ligand. After photolabeling, digestion, and affinity (biotin/streptavidin) purification, MALDI-TOF analysis provides single peaks (with a characteristic isotopic pattern) for species nonrelated to the ligand−receptor covalent complex and double peaks separated by the difference in mass between the deuterated and nondeuterated species (singly charged ion) that are specific of the complex. This easy and reliable reading out of the relevant species requires neither tedious controls nor high numbers of repetitive experiments. In addition, the proteolytic stability of the ligand can also be followed, depending on the localization of the deuterium atoms in the peptide sequence relative to the position of the photoactivable amino acid and the N-terminal biotin sulfone. Nevertheless, the number of deuteriums incorporated in the peptide sequence has to be high enough to prevent too much interference between the isotopic patterns of deuterated and nondeuterated species. Typically, a labeling using five or six deuterium atoms is well adapted for peptides with mass of <3000 u.

The potential of this strategy combining affinity and isotope tags was investigated by photolabeling the hNK-1 receptor expressed in Chinese hamster ovary (CHO) cells. For this purpose, photoactivable analogues of SP were synthesized, i.e., [D_3]-Bapa[(*p*Bzl)Phe^5, Met(O_2)^{11}]SP, [D_3]-Bapa[(*p*Bzl)Phe^5, [D_2]-Gly^9, Met(O_2)^{11}]SP, Biot(O_2)−([D_2]Gly)$_3$-[(*p*Bzl)Phe^5, Met(O_2)^{11}]-SP and the nondeuterated analogues Bapa[(*p*Bzl)Phe^5, Met(O_2)^{11}]-SP and Biot(O_2)−(Gly)$_3$-[(*p*Bzl)Phe^5, Met(O_2)^{11}]SP, all incorporating *p*-benzoyl-L-phenylalanine ((*p*Bzl)Phe) at position 5 (see Figure 1 for chemical modifications). [D_3]-Bapa[(*p*Bzl)Phe^5, Met(O_2)^{11}]SP corresponds to the trideuterated peptide (D_3), [D_3]-Bapa[(*p*Bzl)-Phe^5, [D_2]Gly^9, Met(O_2)^{11}]SP to the pentadeuterated peptide (D_5), and Biot(O_2)−([D_2]Gly)$_3$-[(*p*Bzl)Phe^5, Met(O_2)^{11}]SP to the hexadeuterated analogue (D_6). The pentadeuterated analogue [D_3]-Bapa[(*p*Bzl)Phe^5, [D_2]Gly^9, Met(O_2)^{11}]SP has two deuterated positions located on each side of the photoactivable amino acid.

EXPERIMENTAL SECTION

Syntheses. (a) Oxidation of (d)-Biotin Sulfone. (d)-Biotin (2.44 g, 10 mmol) in suspension in acetic acid (30 mL) was stirred at room temperature for 65 h in the presence of H_2O_2 (10 mL, 35% in water). Dissolution was first observed within a few hours, and then (d)-biotin sulfone slowly precipitated. This precipitate was filtered off, washed twice with diethyl ether, and dried in vacuo. (d)-Biotin sulfone was obtained as a white solid: 2.34 g (85% yield), mp >260°, R_f (TLC media, $CHCl_3$/CH_3OH/CH_3COOH 9/1/0.5, *p*-dimethylaminocinnamaldehyde detection[10]) 0.2; ^{1}H NMR (500 MHz, $^2H_6$-DMSO) δ 1.4−1.5 (m, 2H, CH_2), 1.5−1.6 (m, 2H, CH_2), 1.6−1.7 (m, 2H, CH_2), 2.25 (t, J = 7.6 Hz, 2H, CH_2-CO), 3.0 (d, 1H, J = 14.5 Hz, H_9), 3.2 (q, 1H, H_6), 3.3 (m, 3H, H_9 + H_2O), 4.4 (m, 2H, H_7, H_8, J $H_{7,8}$ = 9.8 Hz), 6.6 (s, 1H, NH), 6.7 (s, 1H, NH); ESI(+) m/z 277 [M + H^+], ESI(−), m/z: 275 [M − H^-].

(b) Deuteration of (d)-Biotin Sulfone at Carbons 6 and 9. A 1 N NaOD solution was prepared by dropwise additions of D_2O (19 mL) to Na (437 mg, 19 mmol). (d)-Biotin sulfone (1.05 g, 3.8 mmol) was added to the NaOD solution, and the resultant

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Table 1. Summary of Peptide Syntheses and Analytical Data

peptides	mass spectrometry[a] MH+ calc	mass spectrometry[a] MH+ meas	retention time (min)[b] (d)-biotin sulfone	retention time (min)[b] epimer[c]	purity % (yield %)[b] (d)-biotin sulfone	purity % (yield %)[b] epimer[c]
Bapa[(*p*Bzl)Phe^5, Met$(O_2)^{11}$]SP	1859.90	1859.94 ± 0.06	8.2		97.2 (46)	
[D_3]-Bapa[(*p*Bzl)Phe^5, Met$(O_2)^{11}$]SP	1862.92	1862.96 ± 0.06	8.5[d]	9.01[e]	98.0 (12)[f]	96.8 (12)[f]
[D_3]Bapa[(*p*Bzl)Phe^5, [D_2]Gly^9, Met$(O_2)^{11}$]SP	1864.93	1864.93 ± 0.06	8.23[d]	8.88[e]	97.1 (20)[f]	96.1 (18)[f]
Biot(O_2)−([D_2]Gly)$_3$-[(*p*Bzl)Phe^5, Met$(O_2)^{11}$]SP	1937.93	1937.89 ± 0.06	7.1		97.3 (70)	
Biot(O_2)−(Gly)$_3$-[(*p*Bzl)Phe^5, Met$(O_2)^{11}$]SP	1931.89	1931.89 ± 0.06	6.9		98.0 (53)	

[a] Calculated and measured (MALDI-TOF) masses are both reported. [b] The retention times of the purified peptides were determined with isocratic conditions with 60% B using solvents A (H_2O/0.1% TFA) and B (CH_3CN 60%, H_2O 40%, TFA 0.1%). [c] The epimer of (d)-biotin sulfone has a (*R*) configuration at C_6, the carbon α to the sulfur atom. [d] Peak 1 corresponds to trideuterated (d)-biotin sulfone (NMR analysis, data not shown).[e]Peak 2 corresponds to trideuterated biotin sulfone epimer at C_6, α to the sulfur atom (NMR analysis, data not shown). [f] A fraction containing both diastereomers was also collected, 6 and 10%, respectively.

mixture was stirred 30 min at room temperature. The mixture was neutralized by slow additions of 6 N HCl solution. After filtration, the resulting solution was first desalted on a C_{18} reversed-phase column (0.1% TFA aqueous solution), and then deuterated biotin sulfone was eluted with a solution of acetonitrile in 0.1% TFA (4:6). This elution allowed partial separation of the two deuterated diastereomers of biotin sulfone ((d)-biotin sulfone and its epimer at C_6). [D_3]-(d)-Biotin sulfone eluted first and can be isolated. The following fractions containing both trideuterated (d)-biotin sulfone and its epimer epibiotin sulfone were purified again. Altogether, [D_3]-(d)-biotin sulfone was obtained pure (447 mg), whereas the trideuterated epibiotin sulfone was always obtained as a mixture of both diastereomers (458 mg): R_f (TLC media, $CHCl_3$/CH_3OH/CH_3COOH 9/1/0.5, detection with *p*-dimethylaminocinnamaldehyde); [D_3]-(d)-biotin sulfone 0.17, [D_3]-epibiotin sulfone 0.40; ^{1}H NMR (250 MHz, $^2H_6$-DMSO) for trideuterated-(d)-biotin sulfone δ 1.3−1.8 (m, 6H, $3CH_2$), 2.2 (t, 2H, CH_2CO), 4.3 (m, 2H, H_7, H_8), 6.6 (s, 1H, NH), 6.7 (s, 1H, NH); and for a 1/1 ratio of trideuterated-(d)-biotin sulfone and its epimer δ 1.3−1.8 (m, 6H, $3CH_2$), 2.2 (t, 2H, CH_2CO), 3.93 (d, 1H, J_{AB} = 9.8 Hz, for H_7 of trideuterated epibiotin sulfone), 4.2 (d, 1H, J_{AB} = 9.8 Hz, H_8 of trideuterated epibiotin sulfone), 4.35 (q, 2H, H_7, H_8 of trideuterated-(d)-biotin sulfone), 6.7 (s, 1H, NH), 7.0 (s, 1H, NH); ESI(−) *m*/*z* 278 [M − H^-].

To firmly establish the stereochemistry of the trideuterated epibiotin sulfone, the exchange reaction was also performed on biotine sulfone in 1 N NaOH to measure all coupling constants of the different protons and to assign the stereochemistry at C_6 from the Karplus equation. Complete assignments of the NMR spectrum gave the following resonances and coupling constants for epibiotin sulfone: ^{1}H NMR (500 MHz, $^2H_6$-DMSO) δ 1.4−1.5 (m, 2H, CH_2), 1.5−1.6 (m, 2H, CH_2), 1.6−1.7 (m, 2H, CH_2), 2.20 (t, *J* = 7.6 Hz, 2H, CH_2CO), 3.17 (m, 2H, H_6 and H_9), 3.53 (m, 1H, *J* $H_{8,9'}$ = 7.4 Hz, $H_{9'}$), 3.94 (m, 1H, *J* $H_{6,7}$ = 6.6 Hz and *J* $H_{7,8}$ = 9.8 Hz, H_7), 4.30 (m, 1H, *J* $H_{8,9}$ = 5.8 Hz, *J* $H_{9,9'}$ = 14.2 Hz, H_8), 6.7 (s, 1H, NH), 7.0 (s, 1H, NH).

(c) Peptide Syntheses. Peptides syntheses were carried out on an ABI model 431A peptide synthesizer using t-Boc strategy and starting from a α-*p*-methylbenzhydrylamine resin (substitution 0.9 mmol/g of resin). All Nα-Boc amino acids (10-fold excess) were assembled using dicyclohexylcarbodiimide and 1-hydroxybenzotriazole as coupling reagents; only a 5-fold excess of Boc-Met(O_2), Boc-[D_2]Gly, and Boc-(*p*Bzl)Phe were used. After coupling Boc-aminopentanoic acid (10-fold excess) and removal of the Boc-protecting group, (d)-biotin sulfone or the mixture of trideuterated biotin sulfone diastereomers was coupled manually overnight (5-fold excess) after dissolution in *N*-methylpyrrolidone and activation by dicyclohexylcarbodiimide and 1-hydroxylbenzotriazole. Coupling efficiency was monitored with the Kaiser test. After completion, the peptidyl-resin was transferred into the Teflon vessel of an HF apparatus and the peptide was cleaved by treatment with 1.5 mL of anisole, 0.25 mL of dimethyl sulfide, and 10 mL of anhydrous HF per gram of peptide-resin for 1 h at 0 °C. After evaporation of HF in vacuo, the resin was first washed (3×) with diethyl ether and then eluted (3×) with acetic acid/H_2O (1:1). Lyophilization of the extract led to crude peptides, which were purified by reversed-phase HPLC with a C_8 (7 μm) Symmetry Prepcolumn (7.8 × 300 mm); separation was accomplished using various acetonitrile gradients in aqueous 0.1% TFA at a flow rate of 6 mL/min with UV detection fixed at 220 nm. Before pooling, the purity of the collected fractions was ascertained by analytical HPLC, with a C_8 (10 μm) Lichrospher 100 RP column (4 × 250 mm) in isocratic mode at a flow rate of 1.5 mL/min with UV detection at 220 nm. Separation of the diastereomers of the two following peptides [D_3]-Bapa[(*p*Bzl)Phe^5, Met$(O_2)^{11}$]SP and [D_3]-Bapa[(*p*Bzl)Phe^5, [D_2]Gly^9, Met$(O_2)^{11}$]SP was achieved by preparative HPLC. Further data on synthesized peptides are summarized in Table 1.

Biotin−Streptavidin Affinity. 2-(4′-Hydroxyazobenzene)-benzoic Acid (HABA) Titration Curves.[11] The titrations were performed at 500 nm by successive additions of 4 μL of biotin or biotinylated peptide solutions (100 μM) to 300 μL of streptavidin (3.03 μM) and HABA (206 μM) solution in 0.2 M NH_4OAc, 0.2% NaN_3 (w/v), pH 7.0, as described.[11] The absorbencies, corrected for dilution, were plotted versus the amount of biotin.

Streptavidin-Coated Magnetic Beads. A solution consisting of a 1:1 ratio mixture (10 pmol/μL) of deuterated and nondeuterated peptides was prepared in 1 mL of 50 mM Tris-Cl (pH 7.4) containing 0.1% bovin serum albumin. Samples were gently stirred for 1 h with 100 μg of streptavidin-coated magnetic beads before washes and MALDI-TOF analysis as described previously.[3]

Cell Culture and Membrane Preparation. CHO cells expressing the human NK-1 receptor (6 pmol/mg of proteins) were cultured in DMEM supplemented with 100 IU/mL penicillin, 100 IU/mL streptomycin, and 10% fetal calf serum at 37 °C in a humidified atmosphere of 5% CO_2. Membranes were prepared as

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described[4] and stored in 50 mM Tris-Cl (pH 7.4), 1 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM phenylmethanesulfonyl fluoride (PMSF), 5 μg/mL soybean trypsin inhibitor (STI), and 10% glycerol at −80 °C.

Photolabeling Experiments.[4−6] Membranes (1−2 mg of proteins) from CHO cells expressing the human NK-1 receptor were incubated for 10 min at room temperature with the peptide mixture (used at a concentration of 10-fold their affinity —K_i— for the NK-1 receptor). The membrane preparation was then irradiated on ice at 365 nm at a distance of 6−10 cm for 40 min. After irradiation, the sample was centrifuged for 2 min at 13 000 rpm, washed with Tris-Cl buffer, and centrifuged again. Finally, photolabeled membranes were incubated for 6 h at room temperature in a denaturation buffer consisting of 17 mM dithiothreitol and 3% SDS in 30 μL of 50 mM Tris-Cl (pH 8.0).

Trypsin Digestion. After denaturation, photolabeled membranes were digested for 15 h at 22 °C with 100 μg of trypsin in 1 mL of 50 mM NH_4HCO_3 (pH 8.0). Digestion was stopped by adding 10 μL of PMSF (0.1 M) and 10 μL of STI (5 μg/mL). The digested sample was then incubated with 100 μg of streptavidin-coated magnetic beads as described previously[4] for 1 h at 4 °C. The sample was then either submitted to further digestion with endo-GluC or analyzed by MALDI-TOF MS.

Endo-GluC Digestion on Streptavidin-Coated Magnetic Beads. After purification and washing,[4] the beads were incubated at 37 °C for 15 h with 20 μg of endo-GluC in 20 μL of 100 mM Tris-Cl (pH 7.8). Digestion was stopped by the addition of 10 μL of PMSF (0.1 M) and 10 μL of STI. A 100-μg aliquot of streptavidin-coated magnetic beads were again added for 1 h at 4 °C before the washing steps.[4]

CNBr Cleavage. After denaturation of membranes, 100 μL of 70% formic acid was added with a few crystals of CNBr for 15 h at room temperature. After endo-GluC digestion, 100 μL of 70% formic acid was added to the beads. The supernatant—containing the ligand−receptor complex—was transferred to a new tube and incubated with a few crystals of CNBr for 15 h at room temperature. Tubes were kept in the dark under argon atmosphere.

MALDI-TOF Mass Spectrometry. Mass spectra were acquired on a MALDI-TOF Voyager Elite (Applied Biosystems, Framingam, MA) in the reflector mode, and the delayed extraction conditions were optimized for the working range (m/z < 4500), and typical resolution $m/\Delta m \sim 5000$ was achieved. Saturated solution of α-cyano-4-hydroxycinnamic acid in acetonitrile/water (0.1% TFA) (4:1, v/v) was freshly prepared, and 3 μL was added to the streptavidin-coated magnetic beads for 10 min. Two different sample preparations were studied. The first one corresponds to the elution of the peptide solution by blocking the beads with a magnet, as previously described,[3] and 1 μL is deposited on the target holder. The second one was the direct deposit of 1 μL of matrix containing beads. Both preparations generally gave similar results in terms of sensitivity and resolution, although the second one provided more durable and intense signals during acquisition of the mass spectra.

Due to the very small amounts of peptides, the signal-to-noise ratio was not sufficiently high if the TOF mass spectrum was averaged on a limited number of the laser shots. Thus, averaging 10−20 mass spectra, each of 256 laser shots, was needed to get a reliable mass spectrum with a good statistic on the isotope patterns. Comparing the ion intensities of the ligand from controlled experiments with known matrix/analyte ratios and from photolabeling experiments evidenced that, in these last experiments, peptides in the 1−10-fmol range are expected on the target. The mass spectra were externally calibrated based on the protonated molecules of both the matrix and a known peptide (neurotensin or [Tyr^8]SP). Alternatively, an internal calibration can also be done with the peptide ligand.

A custom-made program was developed (Visual Basic V6.0) to deduce the isotopic pattern of the doublet taking into account the unlabeled/labeled molar ratio for comparison with experimental data. Finally, the Protein Analysis WorkSheet software (PAWS, freeware edition, Proteomics, http://www.proteomics.com) was used to research matching masses between the mass spectrometry data and the hNK-1 receptor sequence.

RESULTS

[D_3]-(d)-Biotin sulfone, obtained by H_2O_2 oxidation in acetic acid, was prepared by hydrogen−deuterium (H−D) exchange in 1 N NaOD solution. According to this procedure, the three protons α to the sulfone were readily and totally H−D exchanged, since no resonance α to SO_2 was observed in the NMR spectrum of the crude material. After desalting on a C_{18} reversed-phase column, elution allowed us to recover only [D_3]-(d)-biotin sulfone as a pure enantiomer after two purification steps, its structure being determined by comparison with the biotin sulfone NMR spectrum. The structure of the second eluted molecule, which could not be isolated as a pure isomer but coeluted with trideuterated (d)-biotin sulfone, has also been established from NMR experiment and corresponds to [D_3]-epibiotin sulfone. The assignments of all resonances of trideuterated biotin sulfone diastereoisomers can be attributed by ^{1}H NMR analysis in D_6-DMSO of the diastereoisomeric mixture, since the chemical shifts and J^3 coupling constant patterns of the junction protons of the two diastereoisomers are completely different. To unambiguously attribute the absolute configuration at C_6 of the second eluted molecule, epibiotine sulfone was prepared by treating biotin sulfone in 1 N NaOH.

Diastereomeric mixtures of trideuterated biotinylated peptides were prepared with the diastereomeric mixture of trideuterated (d)-biotin and epibiotin sulfones, due to the rather low recovery yield (30%) of pure [D_3]-(d)-biotin sulfone starting from (d)-biotin sulfone. Both peptide diastereomers of [D_3]-Bapa[(*p*Bzl)Phe^5, Met-$(O_2)^{11}$]SP and [D_3]-Bapa[(*p*Bzl)Phe^5, [D_2]Gly^9, Met$(O_2)^{11}$]SP were successfully separated by preparative HPLC. The absolute configuration at C_6 of the biotin sulfone moiety of each isomeric peptide was also unambiguously attributed by 500-MHz ^{1}H NMR analysis of both diastereomers (H_7, H_8 chemical shifts and coupling patterns, data not shown). In both cases, the trideuterated (d)-biotin sulfone-substituted peptides eluted first, before the epimeric trideuterated biotin sulfone-substituted isomer. Thus, in the following sections, peak 1 of [D_3]-Bapa[(*p*Bzl)Phe^5, Met$(O_2)^{11}$]-SP and [D_3]-Bapa[(*p*Bzl)Phe^5, [D_2]Gly^9, Met$(O_2)^{11}$]SP will correspond to the (d)-biotin sulfone isomer, while peak 2 of these peptides will correspond to epibiotin sulfone.

Streptavidin quenching and subsequent elution of mixtures of these diastereomeric peptides, [D_3]-Bapa[(*p*Bzl)Phe^5, Met$(O_2)^{11}$]-SP or [D_3]-Bapa[(*p*Bzl)Phe^5, [D_2]Gly^9, Met$(O_2)^{11}$]SP, by streptavidin-coated magnetic beads were analyzed by MALDI-TOF mass

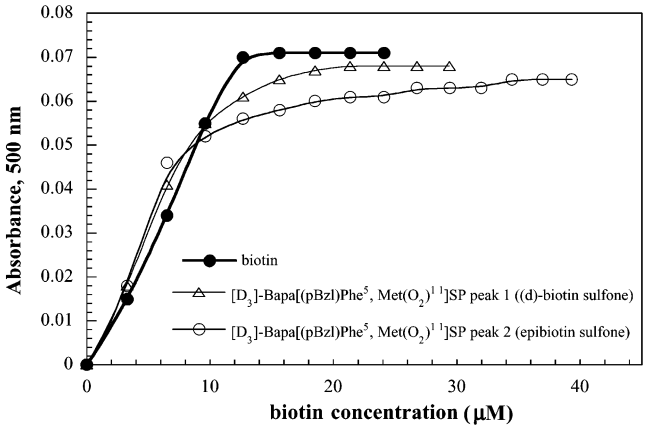

Figure 2. Displacement curves of HABA from streptavidin by successive additions of biotin, [D_3]-Bapa[(*p*Bzl)Phe^5, Met(O_2)^{11}]SP peak 1 (biotin sulfone) or [D_3]-Bapa[(*p*Bzl)Phe^5, Met(O_2)^{11}]SP peak 2 (epibiotin sulfone). Absorbance is corrected for dilution as $A_{ox}V_o - A_{ix}V_i$ (with A_o corresponding to absorbance of the complex HABA/streptavidin alone with a volume V_o and A_i to absorbance of the complex in the presence of a volume V_i of biotin or biotin sulfone-containing peptide) and plotted versus the added biotin.

spectrometry, as previously described.[2−5] Incubation of [D_3]-Bapa-[(*p*Bzl)Phe^5, Met(O_2)^{11}]SP (peak 1) and Bapa[(*p*Bzl)Phe^5, Met-(O_2)^{11}]SP in a 1:1 ratio allows a 1:1 recovery of both nondeuterated and trideuterated peptides. The behavior was different with the trideuterated epibiotin sulfone-substituted peptides. Incubation with streptavidin-coated beads and then elution of [D_3]-Bapa-[(*p*Bzl)Phe^5, Met(O_2)^{11}]SP (peak 2) and Bapa[(*p*Bzl)Phe^5, Met-(O_2)^{11}]SP in a 1:1 ratio shows a preferential recovery (1:2 ratio) of the nondeuterated peptides, that is, the peptide bearing (d)-biotin sulfone versus the peptide with the epibiotin sulfone at the N-terminus. The epibiotin sulfone-substituted peptides could never be totally quenched even with a large excess of streptavidin-coated magnetic beads. Similar results were obtained with incubation performed with the diastereomers (peak 1 and peak 2) of [D_3]-Bapa[(*p*Bzl)Phe^5, [D_2]Gly^9, Met(O_2)^{11}]SP and Bapa[(*p*Bzl)Phe^5, Met(O_2)^{11}]SP. The lower affinity for streptavidin of the epibiotin sulfone moiety was also ascertained by titration of streptavidin in solution with HABA (Figure 2). Moreover, as previously observed,[11,12] although avidin and streptavidin are supposed to present four independent/equivalent binding sites, the "third and fourth" binding sites of the tetrameric avidin did not bind the biotinylated peptides, without spacer[13] between (d)-biotin sulfone and the first amino acid, with the same avidity as the two other ones. Even in the presence of an aminopentanoic spacer between biotin sulfone and the peptide sequence, the lower affinity for streptavidin is still observed with peak 1 of [D_3]-Bapa[(*p*Bzl)Phe^5, Met(O_2)^{11}]SP for approximatively one-fourth of the streptavidin binding sites. With peak 2 (epibiotin sulfone), this difference in affinity occurred already at midpoint addition, that is for half of the binding sites of the tetrameric streptavidin. Consequently, diastereomeric mixtures of trideuterated biotinylated peptides cannot be used without prior separation for photolabeling experiments, since a 1:1 ratio recovery of nondeuterated and deuterated peptides is mandatory for nonambiguous attribution of peaks specific for the peptide−receptor covalent complex by MALDI-TOF mass spectrometry. With the peptides modified by a

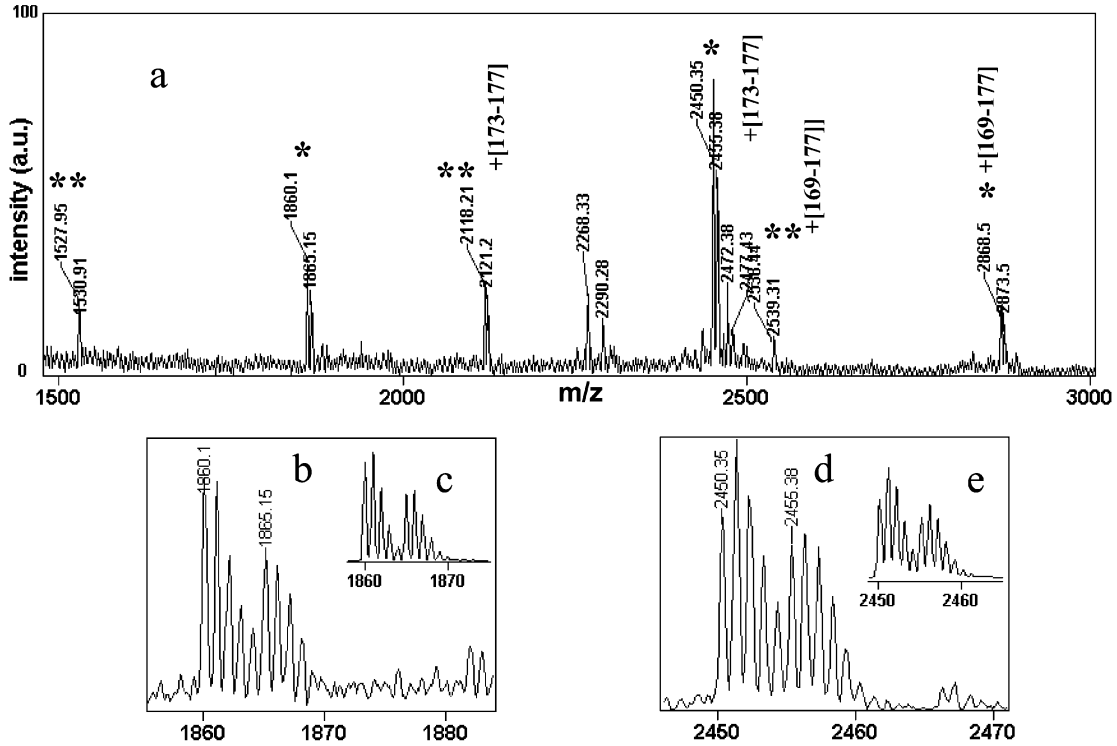

Figure 3. (a) Partial positive ion MALDI-TOF mass spectrum of the peptides after photolabeling, combined digestion (trypsin and endoprotease V8), and affinity extraction using the isotope-unlabeled Bapa[(*p*Bzl)Phe^5, Met(O_2)^{11}]SP and D_5–labeled ligand (molecular ratio 1:0.6); (b) region around the unbound ligand; (c) calculated isotope pattern of the ligand; (d) region of the ligand covalently attached to the fragment of the receptor [173−177]; and (e) its expected isotope pattern. All ions are protonated molecules, and the *m*/*z* values refer to those of the first peak of the isotope pattern. Relevant peaks are marked with asterisks for those corresponding to (∗) the intact ligands (doublets with a *m*/*z* shift of 5) and to (∗∗) the partially digested ligand (doublets with a *m*/*z* shift of 3). Intensity is given in arbitrary units (au).

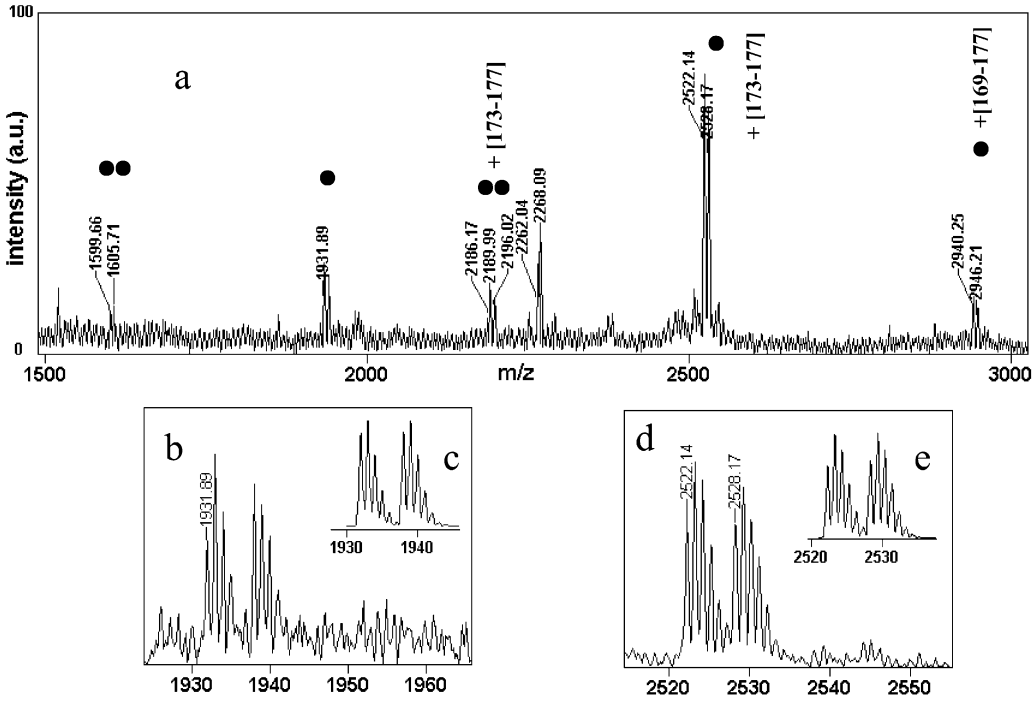

Figure 4. (a) Partial positive ion MALDI-TOF mass spectrum of the peptides after photolabeling, combined digestion (trypsin and endoprotease V8), and affinity purification using the isotope-unlabeled Biot(O_2)−$(Gly)_3$-[(*p*Bzl)Phe^5, Met(O_2)^{11}]SP and D_6-labeled ligand (molecular ratio 1:1); (b) region around the unbound ligand; (c) calculated isotope pattern of the ligand; (d) region of the ligand covalently attached to the fragment of the receptor [173−177]; and (e) the corresponding expected isotope pattern. All ions are protonated molecules, and the *m*/*z* values refer to those of the first peak of the isotope pattern. Relevant peaks are marked with one full circle for those involving the intact ligand (doublets with a *m*/*z* shift of 6) and two for the partially digested ligand (doublets with a *m*/*z* shift of 6). Intensity is given in arbitrary units (au).

p-benzoylphenylalanine in position 5 of SP, the two biotinylated diastereomers could be separated by preparative HPLC, but total recovery of both diastereomers was not quantitatively achieved. Thus, it turned out that incorporation of bideuterated glycine for labeling the peptide would be easier to use than deuterated biotin alone. In the case of SP, [D_2]-glycine can be introduced in the sequence of SP (position 9); otherwise the aminopentanoic spacer can be replaced by three deuterated glycines. Thus, we synthesized and purified both diastereomers of [D_3]-Bapa[(*p*Bzl)Phe^5, [D_2]Gly^9, Met(O_2)^{11}]SP (D_5) and also Biot(O_2)−([D_2]$Gly)_3$-[(*p*Bzl)-Phe^5, Met(O_2)^{11}]SP (D_6). Indeed, introduction of a deuterated reporter in the sequence of the peptide can give an index of the proteolytic stability of the photoactivable peptide during the different incubation steps, before mass spectrometry analysis, while the hexadeuterated spacer may be used with any photoreactive peptide.

Figure 3 shows the MALDI-TOF mass spectrum using a mixture of the unlabeled peptide Bapa[(*p*Bzl)Phe^5, Met(O_2)^{11}]SP and labeled peptide (D_5) in a molar ratio close to unity. The isotope pattern of the unbound ligand (Figure 3b) allows the deduction of the real unlabeled/labeled molar ratio, i.e., 1.6. The recalculated isotope pattern for this experimental 1.6 ratio is given in Figure 3c. From these data, the isotope pattern of the covalently linked peptides can be calculated and compared to the experimental pattern. Peaks showing a doublet with a *m*/*z* shift of 5 are specific for the covalent complex and could be unambiguously assigned (PAWS) to the covalent attachment of the intact ligand to the fragments ^{169}STTETMPSR^{177} and ^{173}TMPSR^{177}, confirming previous results.[6] A good agreement in the isotope pattern is found for the ligand attached to the fragment ^{173}TMPSR^{177}as depicted in Figure 3d (experimental) and Figure 3e (calculated). Double peaks with a *m*/*z* shift of 3 also confirm the degradation of the bound and unbound ligand, since fragment (1−8) contains isotope labeling only through the biotin group (D_3).

The same experiments were done with unlabeled Biot(O_2)−$(Gly)_3$-[(*p*Bzl)Phe^5, Met(O_2)^{11}]SP and the hexadeuterated (D_6) analogue used in a molar ratio of 1:1. A typical MALDI-TOF mass spectrum is given in Figure 4. All the relevant peaks appearing as a doublet, with a *m*/*z* shift of 6, at *m*/*z* 2940.25, 2522.14, 1931.89, 2189.99, and 1599.66 are easily found. They correspond respectively to a protonated molecule consisting of the intact photoreactive ligand linked to the receptor domain [169−177] (mass measured 1008.36, mass expected 1008.46), [173−177] (mass measured 590.25, mass expected 590.29), or alone (*m*/*z* at 1931.89) and to the partially digested ligand (1−8) attached or not to the receptor fragment [173−177]. In this experiment, isotope labeling is only present on the N-terminal region of the ligand. Thus, both the intact and the digested ligands have the same isotope labeling. It must be mentioned that, for these ions, the intensity ratio between the labeled and unlabeled species is close to unity as expected from the initial ligands mixture. Thus, ions shifted by a *m*/*z* of 6 but with very different relative intensities must be considered as irrelevant and due to nonspecific adsorption on the magnetic beads during the purification step. Typically, ions at *m*/*z* 2262.04 and 2268.09 in Figure 4 are assigned to be irrelevant. Indeed, they generally appeared in mass spectra of both photolabeled and control samples (not shown).

The same experiment was also done with combined digestions (trypsin, endoprotease V8, and CNBr) to precisely the site of photoinsertion of the photoreactive ligands within the domain

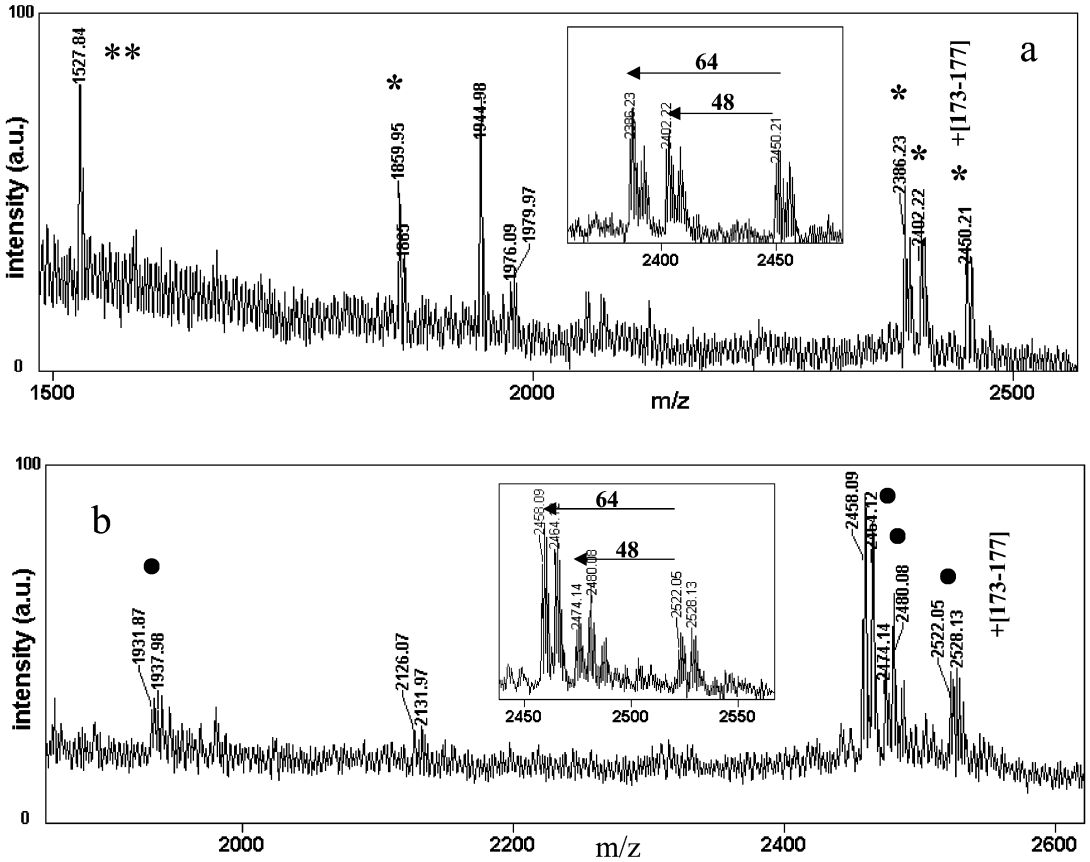

Figure 5. Partial positive ion MALDI-TOF mass spectrum of the peptides after photolabeling, combined digestion (trypsin, endoprotease V8 and CNBr), and affinity purification using (a) the isotope-unlabeled and D_5-labeled ligand mixture (1:0.6) and (b) the isotope unlabeled and D_6 labeled ligand mixture (1:1). Insets show details of the mass spectra involving the intact ligands bound to the receptor fragment [173−177]. All ions are protonated molecules, and the *m*/*z* values refer to those of the first peak of the isotope pattern. Intensity is given in arbitrary units (au).

[173−177] of the hNK-1 receptor (Figure 5). Single peaks at *m*/*z* 1944.98, 1976.09, and 1979.97, generally present in all mass spectra, were assigned to be irrelevant. The MALDI-TOF mass spectra for the unlabeled and D_5 labeled ligands showed new features at *m*/*z* 2386.23 and 2402.22 (Figure 5a), indicating a mass shift of −64 and −48 u with respect to the ligand attached to the [173−177] fragment of the receptor, respectively. Similar shifts were also observed on the new features appearing when a 1:1 mixture of unlabeled and D_6 labeled ligands was used (Figure 5b), indicating that similar products derived from CNBr cleavage were produced. It should be underlined that new species with similar shifts are also detected to a smaller extent. From the doublets profile, these species were attributed to involve the partially digested ligand (1−8) as described above (data not shown). These fragmentations correspond to the covalent linkage of the photoreactive peptide to the $C_\gamma H_2$ of methionine-174 side chain that leads, after CNBr cleavage, to the epoxide/ketone and ethylenic derivatives of the photoprobe.[6,14]

DISCUSSION

It is clear from this study that the two C_6 diastereoisomers in position α to the sulfur atom of biotin (Figure 1) are not recognized with the same affinity by streptavidin. These experiments established that the (*S*) absolute configuration at C_6, is mandatory for high-affinity binding to streptavidin.

Structural analyses of the binding pocket of (d)-biotin in streptavidin or avidin have previously been reported.[16,17] These studies show that ureido oxygen of the biotin molecule forms three hydrogen bonds with the side chain of N^{12}, S^{16}, and Y^{33} (avidin) or N^{23}, S^{27}, and Y^{43} (streptavidin). The tetrahedral oxyanion formed is one of the predominant stabilizing interactions in biotin−avidin or biotin−streptavidin complexes. On the other hand, the presence of the sulfur atom is not a prerequisite for high-affinity binding (10^{-15} M) of biotin to avidin since dethiobiotin and biotin sulfone still bind the protein with dissociation constants of, respectively, 5×10^{-13} and 10^{-13} M.[12] However, the carboxylate of the caproyl side chain of (d)-biotin forms H-bond interactions with S^{73} and S^{75} (avidin) or S^{88} and N^{49} (streptavidin).[18] With the amide group of biotinylated peptide, this stabilizing interaction should not exist. However, the orientation of the valeric side chain, pointing to the opposite direction in epibiotin sulfone relative to (d)-biotin sulfone,

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may result in the loss of stabilizing interactions leading to the decrease observed herein in the binding affinity of epibiotin sulfone-containing peptides for streptavidin.

Proteomics strategies actually combine the use of the so-called light biotin-tag (nondeuterated) with heavy biotin-tag (deuterated) to quantify proteins in control and "abnormal" or experimental situations.[8,9] However, such epimerization of biotin sulfone at the C_6 asymmetric center may only occur at very basic pH ($\geq$11) and fortunately cannot arise in experimental conditions used in proteomics studies.

The second main result of this study shows the suitable use of an equimolar mixture of deuterated and nondeuterated photoreactive peptides to identify specific peaks after photolabeling, purification, and MALDI-TOF analysis of minute amount of covalent complex between the peptide and the protein receptor. The methodology has previously been used without deuterated peptides and was shown to be rather tedious because numerous experiments were required to conclude the identification of the receptor domain.[4−6] Here, doublets in MALDI-TOF mass spectra could in almost all cases be attributed specifically to the covalent complex between the receptor and the photoreactive peptide. Indeed, we have done again the photolabeling of the hNK-1 receptor with the analogue of SP photoreactive in position 5 recently reported,[6] but this time using a 1:1 ratio mixture of penta- or hexadeuterated peptide and the corresponding nonlabeled photoreactive peptides. From one single experiment using the mixture of deuterated and nondeuterated photoreactive peptides, we could conclude the receptor domain was covalently linked while several experiments were required before this could be discerned when only the unlabeled peptide was used. The results obtained in this study confirm that, after CNBr cleavage, the species with a mass shift of −64 and −48 u, with respect to the unlabeled ligand attached to the [173−177] fragment of the receptor previously characterized, is really related to the covalent complex and corresponds to the ethylenic and epoxide/ketone derivatives of this branched peptide.[6,14] Moreover, the use of deuterated peptides confirms that the photoreactive SP analogues are sensitive to trypsin or trypsin-derived chymotrypsin-like activity during long incubation times.[4−7] For example, the pentadeuterated peptide contains three deuteriums on the N-terminal biotin sulfone and two others on Gly^9 in the photoreactive analogue peptide sequence. After trypsin incubation, MALDI-TOF analysis identified doublets shifted by a m/z of 3. These ions correspond unambiguously to branched peptides containing the photoreactive (in position 5) analogue cleaved between Phe^8 and (D_2)Gly^9, meaning that two deuteriums were lost in the purified branched peptide. In contrast, all doublets shifted by a m/z of 5 can unequivocally match a receptor fragment covalently linked to the entire photoreactive peptide. Therefore, previous tedious analyses of mass spectra because of the possible digestion of the photoprobe ligand itself become more easily analyzed in the presence of such "deuterium reporter". This method can be applied to any bioactive peptide containing Gly, Ala, Leu, Phe, Tyr, or Val or amino acids that can be replaced with one of these latter amino acids, without a concomitant loss in peptide affinity. Indeed at least these six amino acids mentioned above are commercially available under deuterated and N-t-Boc-protected forms and may be used in solid-phase peptide synthesis. In addition, a deuterated Gly may be introduced as a spacer arm between biotin and the first amino acid of the peptide sequence. Besides, this methodology can be used for any enzymatic digestion, specific (as with endoprotease V8, trypsin, and Arg-C) or nonspecific (as with pepsin), since only doublets are relevant to the complex and should be analyzed to identify the receptor domain covalently linked.

Finally, such a strategy (use of 1:1 ratio between deuterated and nondeuterated peptides) should be generalized in photolabeling studies using mass spectrometry analysis. However, if this strategy is very useful to identify by MALDI-TOF relevant peaks, it cannot, in any case, identify the amino acid in the receptor covalently linked to the photoreactive ligand. For that purpose, combined digestions as described herein or tandem mass spectrometry is needed. PSD-MALDI-TOF has already been applied to the sequencing of a branched peptide to deduce the residue involved in the covalent linkage.[19] However, the minute amount of branched peptides in our experiments (a few femtomoles) put an obstacle in this way and all attempts to get relevant information in PSD-MALDI were unsuccessful. ESI-MS/MS would be a reliable alternative to get the sequence of the branched peptide. Its sensitivity, as shown in recent proteomic studies when coupled with liquid chromatography,[20,21] lies in the low-femtomole range. Furthermore, this ionization mode is compatible with the affinity purification methods.[22,23] The interpretation of tandem mass spectra would be easier by comparing the fragmentation pattern of unlabeled and labeled relevant branched peptide ions, especially if the deuterium labeling is carried out on both sides of the ligand relative to the photoreactive residue. Finally, LC−MS and LC−MS/MS[20,21] could be interesting alternative techniques to analyze the complex mixture of peptides after photolabeling and digestion without the purification step.

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Received for review May 14, 2003. Accepted September 16, 2003.

AC034512I