N-Tritioacetoxyphthalimide: A New High Specific Activity Tritioacetylating Reagent

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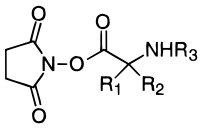
Introduction

The limitations of tritiated acetic anhydride and tritiated acetic acid as acetylating reagents are well known[2−7] and include low tritium content, volatility, and poor chemical selectivity. The activated imido esters (**1**, **2**) have been reported as useful *N*-acylating reagents[8,9] and are highly reactive at 0 °C. The analogous *N*-acetoxy derivatives could be expected to be equally reactive, but have been infrequently utilized.[2,4,7,10−12] Specifically, *N*-acetoxyphthalimide was used to prepare *N*-acetylmuramic acid,[7,11] *N*-(iodoacetoxy)succinimide was used for iodoacetylation of N^6-(6-amino-*n*-hexyl)adenosine 5′-phosphate,[12] and tritiated acetyl derivatives have been produced at low specific activity.[2,10] De Groot[10] reacted [^{14}C]phenylalanyl-soluble-RNA with *N*-acetoxysuccinimide and *N*-(tritioacetoxy)succinimide (no specific activity indicated), and Lindsay[2] used *N*-(tritioacetoxy)succinimide at 21 GBq/mmol to tritioacetylate insulin. In a single example, a high specific activity tritioacetyl group was made by iodoacetylation of muramyl dipeptide with *N*-(iodoacetyl)succinimide and subsequent catalytic tritiodehalogenation.[4] *N*-Succinimidyl [2,3-^{3}H]propionate at very high specific activity has been used to acylate proteins,[13,14] but propionylation may result in appreciable loss of biological activity,[15] may not show selectivity between amino and thiol groups,[16] or may require long reaction times.[17]

R_1, R_2 = H, alkyl, aryl or alkylaryl, R_3 = Protecting group



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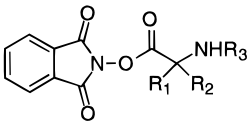

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Our aim was to develop a nonvolatile, stable, and facile tritioacetylating reagent and to demonstrate its use on simple peptides. Accordingly, we made the synthesis of high specific activity *N*-(tritioacetoxy) derivatives of succinimide, phthalimide, and naphthalimide our focus. As our preferred approach, *N*-(tritioacetoxy)phthalimide was prepared by radical dehalogenation of *N*-(iodoacetoxy)phthalimide using high specific activity tributyltin tritide (Scheme 1). This tritiated acetylation reagent was characterized by ^{3}H and ^{1}H NMR spectroscopy and by radio-HPLC. Efficacy of the reagent was investigated by tritioacetylation of several peptides at their N-terminal amino group.

Results and Discussion

Choice of Acetylation Reagent. With the synthesis of high specific activity *N*-tritioacetoxy derivatives of succinimide, phthalimide, or naphthalimide as our goal, our initial investigations centered on *N*-acetoxysuccinimide. Although highly reactive, it is a poorly-chromophoric reagent and therefore difficult to analyze by HPLC. Hence, while the *N*-acetoxysuccinimide reagent may perform very well, we desired two reliable analytical techniques for characterization of the highly tritiated reagent and subsequently concentrated on the phthalimide and naphthalimide derivatives.

We extensively studied the *N*-acetoxyphthalimide precursor and found it to be readily synthesized and to have excellent reactivity, solubility, and chromatographic characteristics. In analogous studies of the naphthalimide derivative, generation of the labeled reagent by the radical dehalogenation approach was unacceptably slow, so that line of investigation was discontinued.

Tritiation of the Acetylation Reagent. Preliminary investigations with catalytic dehalogenation of *N*-(iodoacetoxy)succinimide using H^{3}H gas (10%) in the presence of Pd−C (10%) gave *N*-(tritioacetoxy)succinimide with very poor radiochemical yield (7%) and very low tritium incorporation into the acetoxy group. Analogous tritiation of *N*-(bromoacetoxy)naphthalimide gave the tritiated product with a high chemical yield (85%), but very low specific activity (19 GBq/mmol, *cf.* 106 GBq/mmol theoretical incorporation).

To improve the isotope incorporation, we discontinued the catalytic dehalogenation approach and investigated a radical dehalogenation reaction (Scheme 1). *N*-(Iodoacetoxy)phthalimide was reacted with commercial tributyltin deuteride (100% ^{2}H),[18] and triethylborane was used as the radical initiator. The product *N*-(deuterioacetoxy)phthalimide was obtained in 40% chemical yield with 60% deuterium incorporation. Fresh preparation of tributyltin deuteride[19] was shown by gas chromatography to provide 67% chemical yield of the desired

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(2) Lindsay, D. G.; Shall, S. *Biochem. J.* **1971**, *121*, 737−745.
(3) Wallace, R. J. *Br. J. Nutr.* **1992**, *68*, 365−372.
(4) Baschang, G.; Brundish, D. E.; Hartmann, A.; Stanek, J.; Wade, R. *J. Labelled Compd. Radiopharm.* **1983**, *20*, 691−696.
(5) Barnard, E. A.; Wieckowski, J.; Chiu, T. H. *Nature* **1971**, *234*, 207−209.
(6) Harding, C. R.; Scott, I. R. *Anal. Biochem.* **1983**, *129*, 371−376.
(7) Carroll, P. M. *Nature* **1963**, *197*, 694−695.
(8) Nefkens, G. H. L.; Tesser, G. I. *J. Am. Chem. Soc.* **1961**, *83*, 1263.
(9) Anderson, G. W.; Zimmerman, J. E.; Callahan, F. M. *J. Am. Chem. Soc.* **1964**, *86*, 1839−1842.
(10) de Groot, N.; Lapidot, Y.; Panet, A.; Wolman, Y. *Biochem. Biophys. Res. Commun.* **1966**, *25*, 17−22.
(11) Osawa, T.; Jeanloz, R. W. *J. Org. Chem.* **1965**, *30*, 448−450.
(12) Hampton, A.; Slotin, L. A.; Chawla, R. R. *J. Med. Chem.* **1976**, *19*, 1279−1283.
(13) Fink, D. J.; Gainer, H. *Science* **1980**, *208*, 303−305.
(14) Dolly, O. J.; Nockles, E. A. V.; Lo, M. M. S.; Barnard, E. A. *Biochem. J.* **1981**, *193*, 919−923.
(15) Caras, I. W.; Friedlander, E. J.; Bloch, K. *J. Biol. Chem.* **1980**, *255*, 3575−3580.
(16) Tang, Y. S.; Davis, A.-M.; Kitcher, J. P. *J. Labelled Compd. Radiopharm.* **1983**, *20*, 277−284.
(17) Corringer, P. J.; Durieux, C.; Ruiz-Gayo, M.; Roques, B. P. *J. Labelled Compd. Radiopharm.* **1992**, *31*, 459−468.
(18) Neumann, W. P. *Synthesis* **1987**, 665−683.
(19) Jaiswal, D. K.; Andres, H.; Morimoto, H.; Williams, P. G. *J. Chem. Soc., Chem. Commun.* **1993**, 907−909.

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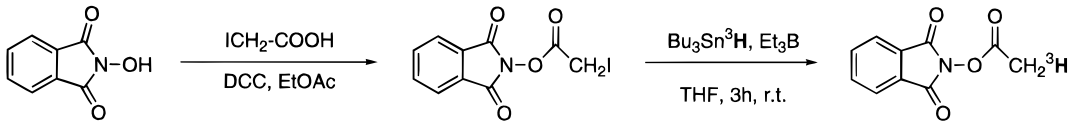
Scheme 1



reagent. To mimic the tritiated reagent and tritiation conditions, this deuterated reagent was used in several *N*-deuterioacetylation reactions.

For the analogous tritiation reactions, high specific activity tributyltin tritide[19] was prepared and used for the radical dehalogenation of the *N*-(iodoacetoxy)phthalimide. Two different batches of the (tritioacetoxy)-phthalimide reagent were prepared. NMR and HPLC analyses confirmed the desired product was present with some byproducts that were formed during the radical dehalogenation reaction. The tin byproducts and other impurities were removed by dissolving the crude reaction mixture in acetonitrile and repeated extraction of the impurities into hexane.[20] Radio-HPLC and proton and tritium NMR analyses of the purified product revealed a radiochemically pure reagent with chemical yields of 38 and 40% and specific activities of 480 and 680 GBq/mmol.

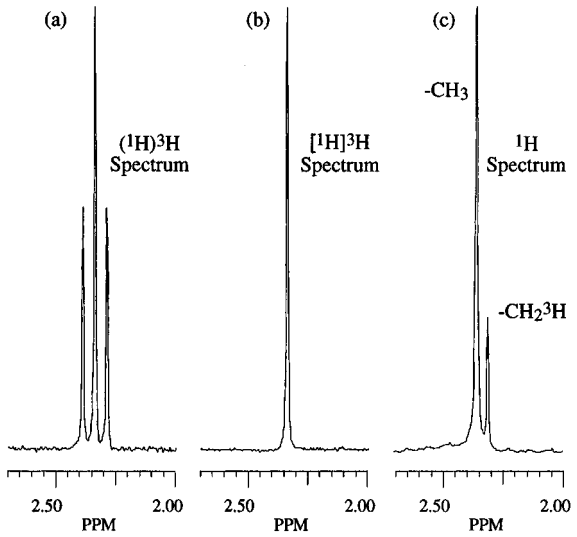
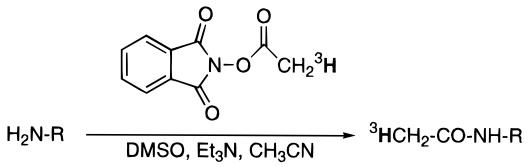


Figure 1. NMR spectra of *N*-(tritioacetoxy)phthalimide: (a) 320 MHz ^{3}H NMR spectrum of *N*-(tritioacetoxy)phthalimide in acetonitrile-d_3 (δ 2.00−2.70 ppm); (b) 320 MHz ^{1}H-decoupled ^{3}H NMR spectrum of *N*-(tritioacetoxy)phthalimide; (c) 300 MHz ^{1}H NMR spectrum of *N*-(tritioacetoxy)phthalimide.

The proton-coupled tritium NMR spectrum of the purified *N*-(tritioacetoxy)phthalimide showed a triplet at 2.36 ppm for the tritioacetoxy group (Figure 1a, J_{HT} = 15.6 Hz), which collapsed to a singlet in the proton-decoupled tritium spectrum (Figure 1b). The proton NMR spectrum of the tritiated sample showed a singlet for the CH_3 species at 2.37 ppm and a doublet for the $CH_2{}^3H$ species at 2.33 ppm. The downfield line of the doublet is obscured by the CH_3 singlet (Figure 1c). Integration of the methyl peaks in this proton spectrum of the tritiated sample allowed calculation of the specific activity of the *N*-(tritioacetoxy)phthalimide at 620 GBq/mmol, in reasonable agreement with HPLC analyses.

Acetylation Reactions. Acetylation of muramic acid,[7] L-cysteine, ACTH (1−4), and neurotensin (8−13) in the presence of triethylamine in dimethyl sulfoxide, dioxane, methanol, or water at room temperature was both rapid and specific. As an example, when 1 equiv of L-cysteine in water was acetylated with 1 equiv of *N*-acetoxyphthalimide in acetonitrile, *N*-acetyl-L-cysteine was the sole product, as shown by NMR and mass spectrometric analyses. Use of 2 equiv of the reagent in the reaction generated *N*,*S*-diacetyl-L-cysteine. For *N*-tritioacetylation reactions (Scheme 2), two peptides, ACTH (1−4) (H_2N-Ser-Tyr-Ser-Met-OH), and neurotensin (8−13) (H_2N-Arg-Arg-Pro-Tyr-Ile-Leu-OH), were selected. The tritioacetylations were carried out by the addition of *N*-(tritioacetoxy)phthalimide in acetonitrile to a solution of the substrate in DMSO under mildly basic conditions, as described fully in the Experimental Section. The tritium NMR spectrum of the *N*-[^{3}H]-acetylated ACTH at the N-terminal serine (specific activity 680 GBq/mmol) showed a single peak at δ = 2.02 ppm. Similarly, a single peak was observed at δ = 2.03 ppm for neurotensin *N*-[^{3}H]-acetylated at the N-terminal arginine. The chemical yield (37%) and specific radioactivity (420 GBq/mmol) of *N*-(tritioacetyl)neurotensin (8−13) were determined by radio-HPLC, starting from the batch of tritiated reagent with S.A. 480 GBq/mmol.

Scheme 2



ACTH (1-4), R = -Ser-Tyr-Ser-Met-OH
Neurotensin (8-13), R = -Arg-Arg-Pro-Tyr-Ile-Leu-OH

The applicability of the reagent for *N*-tritioacetylation reactions was further demonstrated with the labeling of six different peptides (6−17 amino acid residues), prepared by solid-phase techniques. In contrast to the two examples given above, the C-terminus of each peptide was still attached to a (hydroxymethyl)polystyrene (HMP) resin, and all amino acid side chains were protected, with only the N-terminus free for acetylation.[21] After *N*-tritioacetylation, the peptides were released from the resin, deprotected, and purified by radio-HPLC to be used in a peptide precipitation assay.[22] The peptide with the highest specific activity (Ile-Gly-Pro-Gly-Arg-Ala-Phe, 630 GBq/mmol) was also analyzed by tritium NMR spectroscopy and showed a singlet at δ = 2.04 ppm, corresponding to the *N*-(tritioacetyl) group attached to the N-terminal amino acid.

Conclusions

We have demonstrated the synthesis and use of *N*-(tritioacetoxy)phthalimide at high specific activity (up to 680 GBq/mmol) as a new and selective reagent for the

(20) Berge, J. M.; Roberts, S. M. *Synthesis* **1979**, 471−472.

(21) Seligman, S. J. *Anal. Biochem.* **1993**, *211*, 324−325.
(22) Seligman, S. J. *J. Immun. Methods* **1994**, *168*, 101−110.

facile tritium labeling of peptides and other molecules containing a free amino group. The reagent is solid and nonvolatile, and normally 1−1.5 equiv affords rapid and complete acetylation. The chemical and radiochemical stability of this reagent was not specifically studied. However, in one case the tritiated reagent was prepared and stored in acetonitrile, and portions were used for labeling reactions over more than 3 weeks, without any apparent loss of specific activity between reagent and acetylated product. Its facile preparation and analysis make *N*-(tritioacetoxy)phthalimide synthetically very attractive and superior to either tritiated acetic anhydride or tritiated acetic acid as a tritioacetylating reagent. In comparison with *N*-succinimidyl [2,3-^{3}H]propionate, *N*-(tritioacetoxy)phthalimide is unlikely to affect biological activity, gives rapid acylation, and shows high selectivity between thiol and amino groups.

We have also developed a simple and mild method of tritioacetylation of amino acids and peptides in organic solvents (dimethyl sulfoxide, methanol, dioxane) or water. We are currently investigating the utility and selectivity of *N*-(tritioacetoxy)phthalimide in the high specific activity tritioacetylation of several biologically important compounds containing thiol and hydroxyl functional groups.

Similar precursor and labeling chemistry would yield the *N*-(tritioacetoxy) derivatives of succinimide and naphthalimide. If fully developed into tritioacetylation reagents, these offer the promise of a very useful range of solubility and acetylation characteristics. The hazards associated with the generation and use of highly tritiated acetylation or tin-derived reagents should be carefully assessed in planning experiments such as those described in this work.

Experimental Section

General Procedures. Chemical reagents were purchased and purified as previously described.[23] Similarly, mass spectrometric analyses, NMR spectroscopic studies, and liquid scintillation counting details have been published,[23] with the minor change that Opti-Fluor LSC cocktail was used in this project.

High-Pressure Liquid Chromatography. Analytical HPLC was performed on a Chemco Pak silica column. The mobile phase was hexane:ether (75:25) for the *N*-(tritioacetoxy)phthalimide analysis. Peptide analyses were performed on a LC-18 Vydac column, using a gradient mobile phase of acetonitrile/water with 0.1% TFA from 2 to 52% acetonitrile (1−26 min) and flow rate of 1.5 mL/min. UV detection was at 234 and 210 nm on a Hewlett-Packard 1040A diode array spectrophotometer, and radioactivity was monitored by an IN/US β-Ram HPLC flow detector, using a lithium glass scintillant cell with an efficiency of *ca*. 0.5%. The specific radioactivity of the reaction products was determined by comparison of UV standards with the analytical sample, combined with liquid scintillation counting of the isolated HPLC peak effluents.

Synthesis of Unlabeled Acetylation Reagents. A. *N*-Acetoxysuccinimide. This synthesis was identical to a published approach.[24]

B. *N*-Acetoxyphthalimide. Previous syntheses of this compound were not well described.[7,11,25] The current synthesis is similar to early preparations of *N*-(iodoacetoxy)succinimide.[10,12,26] *N*-Hydroxyphthalimide (0.7 g, 4.35 mmol), acetic acid (HOAc, 258 μL, 4.35 mmol), and dicyclohexylcarbodiimide (DCC, 0.85 g, 4.35 mmol) were added to dry ethyl acetate (EtOAc, 125 mL), and the mixture was stirred for 3 h at room temperature and then filtered. The filtrate was dried (Na_2SO_4), and the residue was crystallized from ethanol to give a white solid (0.82 g, 90%): mp 185 °C; ^{1}H NMR δ ($CDCl_3$) 7.76−7.89 (m, 4H), 2.39 (s, 3H).[24]

C. *N*-Acetoxynaphthalimide. The sodium salt of *N*-hydroxynaphthalimide (235 mg, 1 mmol) was suspended in benzene (5 mL), and a solution of acetyl chloride (0.08 mL, 1.14 mmol) in benzene (1 mL) was added dropwise. The reaction was vigorously stirred at room temperature, and after 5 min a white precipitate (NaCl) was formed. TLC of the reaction mixture (hexane:ethyl acetate 90:10) showed a new product (R_f 0.7). The reaction mixture was filtered, and the solvent was evaporated under a stream of nitrogen gas to furnish a pale yellow product (234 mg, 91%): mp 205 °C; ^{1}H NMR δ ($CDCl_3$) 8.64 (d, 2H), 8.29 (d, 2H), 7.79 (t, 2H), 2.47 (s, 3H).

Synthesis of Precursors to the Labeled Acetylation Reagents. A. *N*-(Iodoacetoxy)succinimide. This synthesis was identical to published methods.[10,12,26]

B. *N*-(Iodoacetoxy)phthalimide. This synthesis was based on published preparations of *N*-(iodoacetoxy)succinimide.[10,12,26] *N*-Hydroxyphthalimide (1.4 g, 8.7 mmol), iodoacetic acid (1.6 g, 8.7 mmol), and DCC (1.7 g, 8.7 mmol) were added to dry EtOAc (250 mL), and the mixture was stirred for 5 h at room temperature. The reaction mixture was then filtered, the filtrate was dried, and the residue was crystallized from ethanol to give a white solid (2.15 g, 75%): mp 120 °C; ^{1}H NMR δ (acetone-d_6) 8.01 (m, 4H), 4.32 (s, 2H). Anal. Calcd $C_{10}H_6NO_4I$: C 36.2; H 1.8; I 38.4; Found: C 36.4; H 1.8; I 38.2.

C. *N*-(Iodoacetoxy)naphthalimide. This synthesis was based on published preparations of *N*-(iodoacetoxy)succinimide.[10,12,26] The sodium salt of *N*-hydroxynaphthalimide was acidified and yielded two products, with the major component being the ring-opened product. *N*-Hydroxynaphthalimide (22 mg, 0.1 mmol), iodoacetic acid (18 mg, 0.1 mmol), and DCC (19.5 mg, 0.1 mmol) were added to dry EtOAc (2 mL), and the mixture was stirred for 5 h at room temperature. The reaction mixture was then filtered, the filtrate was dried, and the residue was crystallized from ethanol to give a white solid (27 mg, 69%): mp 184 °C; ^{1}H NMR δ ($CDCl_3$) 8.66 (d, 2H), 8.31 (d, 2H), 7.78 (t, 2H), 4.07 (s, 2H).

Labeling of Acetylation Reagents by Catalytic Dehalogenation. A. *N*-(Tritioacetoxy)succinimide. *N*-(Iodoacetoxy)succinimide (14 mg, 0.05 mmol) was dissolved in EtOAc (1 mL), and Pd−C (10%, 10 mg) and triethylamine (4 μL) were added. The reaction vessel was connected to the vacuum line, and the C−I bond was hydrogenolyzed under 1 atm of 10% tritium gas for 2 h. The reaction was then halted by removal of the tritium gas, and methanol (1 mL) was added and removed by evacuation. The residue was dissolved in EtOAc (1 mL), and the catalyst was filtered off. EtOAc was removed, and THF-d_8 (1 mL) was added for ^{1}H and ^{3}H NMR analyses. The total radioactivity was assessed by liquid scintillation counting as 370 MBq (10 mCi, 7.2%): ^{1}H NMR δ (THF-d_8) 2.23 (s, 3H), 2.71 (s, 4H); [^{1}H]^{3}H NMR (THF-d_8) 2.24 (s, 50%, $-OCOCH_2{}^3H$), 4.05 (s, 50%, unknown).

B. *N*-(Tritioacetoxy)naphthalimide. *N*-(Bromoacetoxy)-naphthalimide (10.3 mg, 0.03 mmol) was dissolved in EtOAc (1 mL), and Pd−C (10%, 10 mg) and triethylamine (5 μL) were added. The reaction vessel was connected to a vacuum line, and the compound was hydrogenated under 1 atm of 10% tritium gas for 2 h. The reaction was then halted by removal of the tritium gas, and methanol (1 mL) was added and removed by evacuation. EtOAc (1 mL) was added, the catalyst removed by filtration, and the product was analyzed by radio-HPLC to give 6.5 mg (85%), specific activity 19 GBq/mmol: ^{1}H NMR δ ($CDCl_3$) 8.65 (d, 2H), 8.31 (d, 2H), 7.80 (t, 2H), 2.47 (s, 2.86H); [^{1}H]^{3}H NMR δ ($CDCl_3$) 2.47 (s); (^{1}H)^{3}H NMR δ ($CDCl_3$) 2.45 (t), J_{HT} = 15.6 Hz.

Labeling of Acetylation Reagents by Radical Dehalogenation. A. *N*-(Deuterioacetoxy)phthalimide. A solution of *N*-(iodoacetoxy)phthalimide (10 mg, 0.03 mmol) in dry THF (0.3 mL) was added dropwise to a mixture of tributyltin deuteride (13 μL, 0.045 mmol) and triethylborane (4 μL, 0.004 mmol) in dry THF (0.7 mL) under a nitrogen atmosphere. The reaction was stirred at room temperature for 3 h. After reaction, the solvent was removed under a flow of nitrogen gas, and the

(23) Than, C.; Morimoto, H.; Andres, H.; Williams, P. G. *J. Org. Chem*. **1995**, *60*, 7503−7507.
(24) Grochowski, E.; Jurczak, J. *Synthesis* **1977**, 277−279.
(25) Neunhoeffer, O.; Gottschlich, R. *Liebigs Ann. Chem*. **1970**, *736*, 100−109.
(26) Slotin, L. A.; Hampton, A. *Nucleic Acid Chem*. **1978**, *2*, 843−846.

residue was dissolved in acetonitrile (1 mL) and extracted with hexane (1 mL, 5×). HPLC analysis of the acetonitrile phase showed the desired product (2.36 mg, 38%): mp 190 °C; *m/z* 206 (60% D); $[^1H]^2H$ NMR δ ($CHCl_3$) 2.40 (s), $(^1H)^2H$ NMR 2.39 (t).

B. *N*-(Tritioacetoxy)phthalimide. *N*-(Iodoacetoxy)phthalimide (20 mg, 0.06 mmol) was dissolved in dry THF (0.6 mL), and this solution was added to freshly prepared tributyltin tritide[19] (0.09 mmol) and triethylborane (8 μL, 0.008 mmol) in dry THF (1.4 mL) under N_2. The reaction mixture was then stirred at room temperature for 3 h, after which time the solvent was removed under a flow of nitrogen gas. The crude reaction product was dissolved in acetonitrile and extracted with hexane (1 mL, 5×). The acetonitrile was then removed under a flow of nitrogen gas and the residue dissolved in deuteriated acetonitrile (1 mL). Radio-HPLC analysis showed 40% yield of the desired product with a specific activity of 640 GBq/mmol: $[^1H]^3H$ NMR δ (CD_3CN) 2.36 (s); $(^1H)^3H$ NMR 2.35 (t), $J_{HT} = 15.6$ Hz.

C. *N*-(Deuterioacetoxy)naphthalimide. *N*-(Iodoacetoxy)-naphthalimide (10 mg, 0.026 mmol) was dissolved in dry THF (0.3 mL), and the mixture was added dropwise to a solution of tributyltin deuteride (13 μL, 0.045 mmol) and triethylborane (4 μL, 0.004 mmol) in dry THF (0.7 mL) under nitrogen. After the reaction was stirred at room temperature for 4 h, monitoring by TLC (hexane:ethyl acetate 5:1) showed only 10−20% product. The reaction was continued for an additional 12 h, after which time the solvent was removed under nitrogen and the residue was dissolved in $CDCl_3$ for ^1H NMR analysis. The ratio of the product to starting material was estimated to be approximately 4:1: ^1H NMR δ ($CDCl_3$) 8.66 (d, 2H), 8.31 (d, 2H), 7.78 (t, 2H), 2.47 (s, 2H); *m/z* 256 (64% D).

General Procedure for the *N*-Tritioacetylation of Peptides. Freshly prepared *N*-(tritioacetoxy)phthalimide (0.58 mg, 2.8 μmol, 480−680 GBq/mmol) was dissolved in EtOAc or acetonitrile (250 μL). This solution was added to a mixture of the peptide (2 μmol) in DMSO (250 μL) and triethylamine (5 μL). The mixture was vortexed at room temperature for 1 h. A slight yellow to orange color was observed after the reaction was finished. The solvents were removed by lyophilization, the residue was dissolved in deuteriated water (300 μL) and filtered, and the filtrate was analyzed by radio-HPLC and tritium NMR spectroscopy. *N*-[^{3}H]Acetyl-ACTH (1−4): $[^1H]^3H$ NMR δ (D_2O) 2.02 (s); $(^1H)^3H$ NMR δ (D_2O) 2.02 (t). *N*-[^{3}H]Acetylneurotensin (8−13): $[^1H]^3H$ NMR δ (D_2O) 2.03 (s); $(^1H)^3H$ NMR δ (D_2O) 2.03 (t).

General Procedure for the *N*-Tritioacetylation of HMP-Peptides. The H_2N-Ile-Gly-Pro-Gly-Arg-Ala-Phe-HMP (8.3 mg, 3.96 μmol) was suspended in DMSO (100 μL) in a conical vial, and triethylamine (2 μL) was added. *N*-(Tritioacetoxy)phthalimide (640 GBq/mmol, 3.65 GBq, 1.2 mg, 5.7 μmol) in EtOAc (100 μL) was prepared and added to the HMP-peptide in DMSO. The reaction vessel was vortexed at room temperature for 30 min. The supernatant (200 μL) was separated and the residue was then centrifuged at 15000*g* for 2 min and washed and centrifuged alternately with *N*-methylpyrrolidine (0.5 mL) and dichloromethane:methanol (1:1, 0.5 mL) for a total of six washes. The residue was lyophilized for 10 min, and the *N*-[^{3}H]-acetylated peptide was deprotected and released from the resin by the addition of thioanisole (10 μL), ethanedithiol (10 μL), and trifluoroacetic acid (80 μL) while it was intermittently vortexed for 2 h. Water (0.5 mL) was added, and the vial was centrifuged to separate the resin. The supernatant was removed, and the pellet was washed with the addition of water (0.5 mL). The combined supernatant was lyophilized overnight (the usual ether precipitation step[21] was omitted because of the availability of high vacuum apparatus). The resultant peptide was dissolved in deuteriated water (0.3 mL) and purified by HPLC to give the desired radiochemically pure peptide with a specific activity of 630 GBq/mmol and chemical yield of 35%: $[^1H]^3H$ NMR δ (D_2O) 2.04 (s); $(^1H)^3H$ NMR δ (D_2O) 2.06 (t).

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