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Combined Fmoc–Alloc Strategy for a General SPPS of Phosphoserine Peptides; Preparation of Phosphorylation-Dependent Tau Antisera

Gideon Shapiro,[a,*] Dieter Büchler, Claudio Dalvit, Peter Frey,[b] Maria del Carmen Fernández, Berta Gomez-Lor, Esteban Pombo-Villar, Urs Stauss,[b] Robert Swoboda[b] and Caroline Waridel[b]

[a]*Preclinical Research, Sandoz Pharma Ltd, CH-4002 Basel, Switzerland*
[b]*Sandoz Research Institute Berne Ltd, CH-3007, Berne, Switzerland*

Abstract—A block method for the solid phase synthesis (SPPS) of serine phosphopeptides has been developed using a combination of Fmoc and Alloc strategies. Alloc-Ser[$PO(OCH_2CH{=}CH_2)_2$]—OH, prepared in a one pot procedure from Alloc-Ser-OH, was introduced at the *N*-terminus of a sequence prepared by standard Fmoc-SPPS. Global cleavage of the allyl ester based protecting groups, followed by coupling of a tripeptide fragment, led to the tau phosphopeptide, **1**. Using tau phosphopeptides a series of phosphorylation state-dependent antisera to human tau protein have been raised. These antisera are valuable tools for studying the tau protein which is found in an abnormal, hyperphosphorylated form in Alzheimer's disease brain. Copyright © 1997 Elsevier Science Ltd

Introduction

The reversible phosphorylation of serine, threonine and tyrosine residues in proteins is one of the most powerful regulatory mechanisms in biology. In the attempts to unravel details of this mechanism, a significant amount of effort has been directed to the development of methodology for the synthesis of phosphopeptides.[1] Some groups have used enzymatic phosphorylation of free peptides, but although in some cases this is an excellent method for preparing phosphopeptides,[2a] in other cases the degree of phosphorylation can be very variable and often poor incorporations of phosphate are obtained.[2b] Additionally, it may be difficult to achieve selectivity in the phosphorylation in peptides containing more than one residue capable of being stably phosphorylated. We have been particularly interested in the practical synthesis of serine-threonine phosphopeptides of significant length and functional complexity for specific biological research applications. Therefore, we required an efficient solid phase method which would enable selective incorporation of the desired phosphate group. Until recently the most practical method for the solid phase peptide synthesis (SPPS) of serine phosphopeptides involved some method of on-resin phosphorylation[3] of a suitably protected peptide after a standard Fmoc[4] based peptide chain assembly (Fig. 1). In our experience, we have found the on-resin phosphorylation method to be acceptable for the synthesis of certain serine phosphopeptides, but to have significant limitations. In practice, the serine residue to be phosphorylated is incorporated either unprotected, or in a selectively protected fashion.[3a] The free hydroxyl function of the serine residue, in the otherwise fully protected resin bound peptide, is phosphorylated to give a phosphate ester which is subsequently deprotected to give free serine phosphate either upon resin cleavage or in a separate step.[5] Due to their broad applicability, phosphoramidate phosphitylation[6] with 'in situ' oxidation protocols are most frequently employed for the phosphorylation step.[7] On-resin phosphorylation methods suffer from several inherent drawbacks. First, using the preferred phosphoramidate based phosphorylation protocols severely impedes if not precludes incorporation of oxidation sensitive residues, such as cysteine, methionine and tryptophan. Second, the phosphorylation efficiency varies widely depending on the reactivity of the serine hydroxyl function which is highly sequence dependent. This is particularly troublesome since it is also difficult to predict. Indeed, large excesses of phosphorylation reagents and long reaction times are often necessary, and for some peptide sequences we have simply not been able to affect appreciable phosphorylation and recover unreacted peptide. Clearly general, effective SPPS building block methods directly incorporating a serine phosphate equivalent are highly desirable. Preferably, such methods should be based on Fmoc SPPS which is widely practiced and operationally much more facile than Boc SPPS,[8] which is limited to the domain of the highly specialized peptide chemist. Herein, we report the full account[9] of a novel general building block method based on a combination of Fmoc and Alloc[10] peptide synthesis strategies which has been used to prepare an important epitope of the tau phosphoprotein present in Alzheimer's Disease: phosphotau (259–268), H-Lys-Ile-Gly-Ser(PO_3H_2)-Thr-Glu-Asn-Leu-Lys-His-OH (**1**)[11]

Key words: Alloc, Alzheimer's Disease, solid phase phosphopeptide synthesis, tau antisera, tau phosphopeptide.

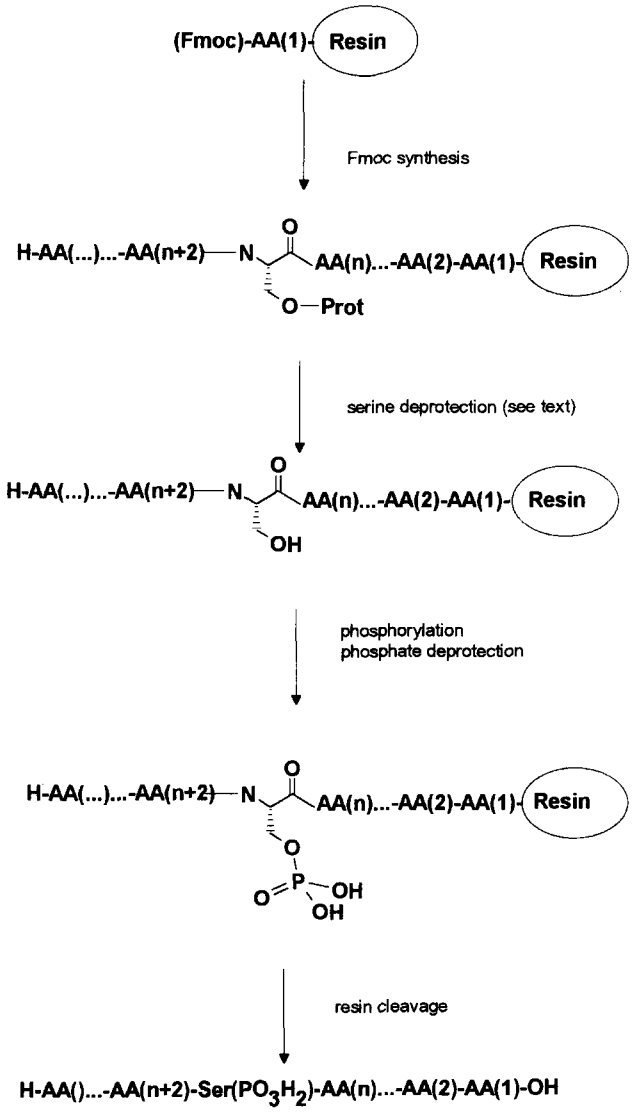


Figure 1. General scheme for Fmoc-based SPSS of phosphopeptides with on-resin phosphorylation.

Using **1** as an immunogen, polyclonal antisera have been raised which selectively recognize this epitope of the tau protein in its phosphorylated state.

Results

At the time we began this work, two accounts of building block approaches for the SPPS of serine phosphopeptides had been reported. One was a Boc synthesis of the tau (139–151) phosphopeptide, H-Lys-Ser-Ser-Pro-Gly-Ser(PO_3H_2)-Pro-Gly-Thr-Pro-Gly-Ser-Arg-NH_2, which required optimization of the phosphate arylester protecting groups on Boc-Ser-[$PO(OAr)_2$]-OH (Fig. 2).[12] The other method made use of α-*N*-allyloxycarbonyl (Alloc) amino acids and *t*-butyl phosphate ester building block Alloc-Ser[$PO(OtBu)_2$]-OH (**5**) and required the efficient method developed by Guibé [$Bu_3SnH\backslash Pd(PPh_3)_4$] for Alloc deprotection.[13] Fmoc-based SPPS methods employing a fully protected serine phosphotriester building block Fmoc-Ser[$PO(OR)_2$]—OH (**2**) are precluded due to the high susceptibility of such phosphate ester functionality to β-elimination[1] under the basic conditions required for Fmoc group removal. As a solution to this problem we reasoned that if the serine phosphate diester functionality could be unmasked to free serine phosphate directly after its incorporation it would become stable to β-elimination. To this end, Alloc-Ser[$PO(OCH_2CH{=}CH_2)_2$]—OH (**6**) was chosen as the key building block with the following strategy. Standard Fmoc-SPPS should be performed up to the phosphoserine residue at which point **6** is introduced. After incorporation of **6** the α-*N*-Alloc group should be cleaved concomitantly with both phosphate allyl ester groups using the efficient deprotection method we had developed for solid phase applications.[14] With the so generated free *N*-terminal serine phosphate on the solid phase stable to β-elimination, extension of the peptide chain might then again be possible via Fmoc-SPPS. The tau phosphopeptide **1**, which we were unable to prepare by post synthetic, 'global' phosphorylation of the free, but apparently inaccessible serine hydroxyl function, was

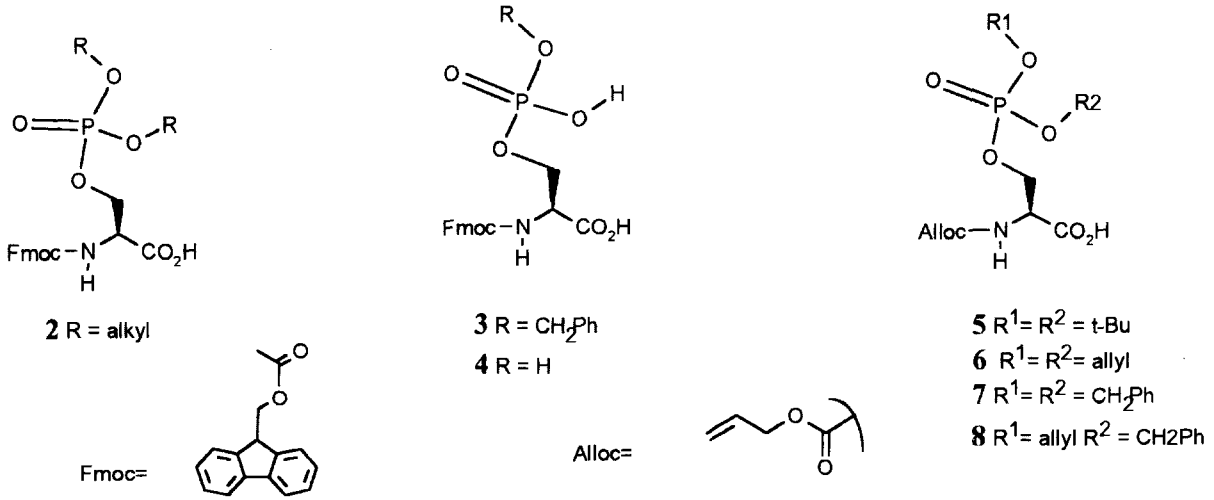
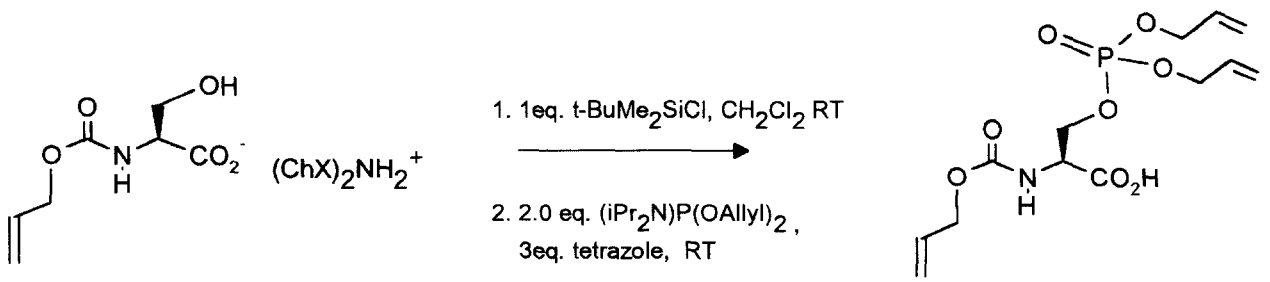


Figure 2. Building blocks for SPSS of phosphopeptides.

the target of the combined Fmoc-Alloc building block strategy as conceived above.[15]

Alloc-Ser[PO(OCH$_2$CH=CH$_2$)$_2$]—OH (**6**) was readily prepared in multigram quantity using the four step procedure described[13] for preparing Alloc-Ser[PO(Ot-Bu)$_2$)]—OH (**5**), which we had previously used to prepare the dibenzyl analogue **7**.[14] It is convenient to store **6** as its stable dicyclohexylamine salt since the free acid is a polymerization sensitive oil. In addition, we have developed a shorter two step preparation of **6** by adapting a known method for the synthesis of tyrosine phosphate esters.[16] In the Bannwarth protocol[16] the acid function of Fmoc-Tyr-OH is transiently protected 'in situ' as the *t*-butyldimethylsilylester, which allows phosphoramidate mediated phosphitylation followed by oxidation to the phosphate stage with aqueous work up yielding the Fmoc-Tyr(PO$_3$R$_2$)—OH derivatives. In our hands although this method was satisfactory for the preparation of Boc and Fmoc serine phosphate esters, the results with Alloc-Ser-OH were poor. Reasoning that a great part of the difficulty with Alloc-Ser—OH was its low solubility in suitable aprotic solvents, we prepared the corresponding dicyclohexylamine salt Alloc-Ser—OH:DCHA, (**9**), which was soluble in dichloromethane. In a one pot procedure **9** could be converted to Alloc-Ser[PO(OCH$_2$CH=CH$_2$)$_2$]—OH (**6**) in moderate (ca 40%) yield by direct silylation with an additional base in dichloromethane followed by the standard phosphorylation protocol, work up and chromatography (Fig 3). Thus, an expedient route to **6** is available which is preferable for smaller scale application.[17]

Hexapeptide H-Thr(But)-Glu(But)-Asn(Trt)-Leu-Lys-(t-Boc)-His(Trt)-Wang (**10**) was prepared (Fig. 4) by Fmoc-SPPS and subjected to amino acid analysis for a precise determination of the resin loading at this stage of the synthesis. The simple DIC-HOBt coupling of **6** with **10** proceeded smoothly in standard fashion to give **11**. Standard cleavage of a resin sample (TFA:EDT) gave material which was virtually homogeneous by HPLC analysis and corresponded to Alloc-Ser(PO(OAllyl)$_2$)-Thr-Glu-Asn-Leu-Lys-His—OH by FABMS. In contrast, coupling of **6** using benzotriazolyl-tetramethyluronium hexafluorophosphate salt (HBTU)[18] as an activator was not satisfactory perhaps due to side reactions related to β-elimination caused by the excess Hünig base employed for buffering. The following key concomitant deprotection of the Alloc and phosphate allyl ester groups using our method[14] (Me_3SiN_3/$Bu_4NF(3H_2O)$/20 mol% $Pd(PPh_3)_4$ in CH_2Cl_2, 30 min room temperature) followed by resin washing gave excellent results.[19] In previous studies toward solid phase phosphopeptide synthesis with dibenzyl analogue **7**, we had found it necessary to develop this alternative to the Bu_3SnH\Pd(PPh$_3$)$_4$ method for an efficient solid phase Alloc deprotection. We have had consistently good results with this method for several other solid phase deprotections of Alloc and allyl phosphate ester groups. However, we would like also to note that the Fmoc group is not stable under these reaction conditions. In the chain elongation with the next amino acid, Fmoc-Gly—OH, ninhydrin monitoring of the coupling reaction proved unreliable and HPLC analysis after cleavage from a resin sample was required for this purpose. Although the coupling at the *N*-terminal of **12** was sluggish, the reaction could be driven to virtual completion without significant side product formation (HPLC analysis) with a single repetition of the DIC-HOBt coupling procedure (double coupling). With a subsequent synthesis cycle (Fmoc cleavage, coupling) HPLC analysis after cleavage of a resin sample indicated increased side product formation. Thus, we encounter problems of an undetermined nature with the free phosphate which compromise chain extension *via* the standard Fmoc method. It was apparent that the best strategy for phosphopeptide synthesis using our method would involve a minimum of synthetic transformations on the resin bound phosphoserine peptide. To complete the synthesis of **1** we planned a single convergent coupling of the requisite, suitably protected tripeptide fragment onto the free phosphoserine *N*-terminal to be followed immediately by liberation of the product directly from the resin. Boc-Lys(Boc)-Ile-Gly—OH was readily prepared by Fmoc SPPS on the 'super' acid sensitive Sasrin resin[20] and easily purified by chromatography over silica gel. The protected fragment was appended to **12** via a DIC-HOBt mediated double coupling which was required for complete reaction. Cleavage of the product **13** from the resin gave a crude product which demonstrated a single major peak using RP18 HPLC analysis. Purification by semipreparative RP-18 HPLC followed by FABMS analysis indicated the major peak to be the desired phosphopeptide **1**, which was verified



9 **6**

Figure 3. One-pot synthesis of building block **6**.

by NMR studies. The synthesis of **1** has been repeated giving an average purified yield of ca. 30%.

NMR Studies

For more complete characterization, 1-D-WATERGATE[21] and 2-D-HMBC[22] (heteronuclear multiple bond phosphorus-proton correlated) and a series of 1-D-PFG (pulsed field gradient) selective ROE and TOCSY NMR experiments were performed on **1** at 400 MHz in water. The WATERGATE experiment enables one to observe the amide N—H resonances and determine peptide structure in water, the biologically relevant milieu. The spectrum shown in Figures 5 (a) and (b) indicates **1** to be very pure, but to contain a minor component presumed to be the isomeric phosphopeptide having the corresponding D-histidine stereochemistry. The nine amide N—H resonances are clearly accountable and for the most part well resolved. The C2 and C4(5) His signals are clearly discernable and the minor signal at 7.21 ppm is also consistent with a C4(5) His resonance. The potential of Fmoc-His(N$_\tau$-Trt)—OH to racemize has been documented[23] and racemization is known to be a problem in the resin esterification.[24] Although we feel confident with our assignment to the D-His isomer, we did not determine the enantiomeric purity of the purchased Fmoc-His(Trt)-Wang resin and therefore do not know the extent of racemization that might have occurred during the synthesis. The HMBC experiment together with a series of one dimensional ^{1}H selective experiments (Fig. 6) allowed then the unambiguous assignment of the phosphate moiety to the serine residue. Only one phosphorus signal was observed in the HMBC spectra which was coupled to two proton resonances as indicated in Figure 6(b). One dimensional selective ROE (data not shown) and TOCSY experiments have

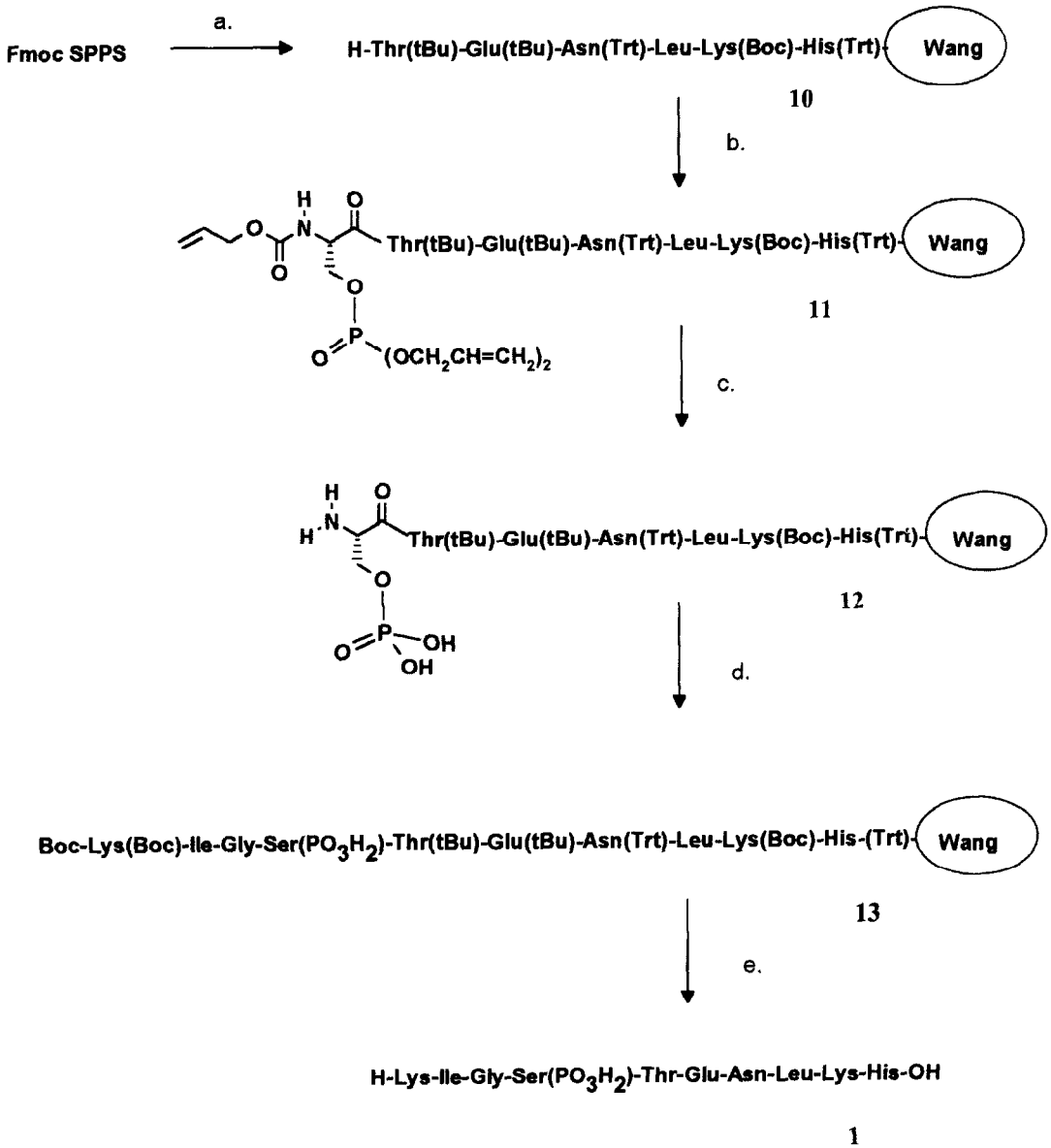


Figure 4. Synthesis of phosphopeptide **1**.

allowed [Fig. 6 (c)] then the assignment of the two resonances to the $C_\beta H$ protons of the serine residue. The NMR spectrum was recorded from the same solution after being kept at room temperature for 1.5 years. Little difference was found indicating the peptide to be quite stable with no tendency for the phosphorus to migrate (e.g. to the adjacent Thr). Finally, no evidence of significant regular and stable secondary structure was found.

Antibody production and immunoreactivity

We have generated polyclonal rabbit antisera against the following phosphorylated partial human tau-441 sequences: htau197–205: N-Tyr-Ser-Ser-Pro-Gly-Ser (PO_3H_2)-Pro-Gly-Thr—OH, htau259–268: N-Lys-Ile-Gly-Ser(PO_3H_2)-Thr-Glu-Asn-Leu-Lys-His—OH (**1**) and htau 389–402: N-Gly-Ala-Glu-Ile-Val-Tyr-Lys-Ser(PO_3H_2)-Pro-Val-Val-Ser-Gly-Asp—OH as well as the corresponding non-phosphorylated sequences.[25] Several antisera were raised to each peptide and titers up to 1:100,000 were obtained. For each phosphopeptide individual antisera were obtained which recognized the peptide against which they had been raised by a factor of up to 1000 better than their respective non-phosphorylated counterpart. All of the antisera also recognized recombinant human tau and native tau, isolated from brains of various species in a phosphoryl-

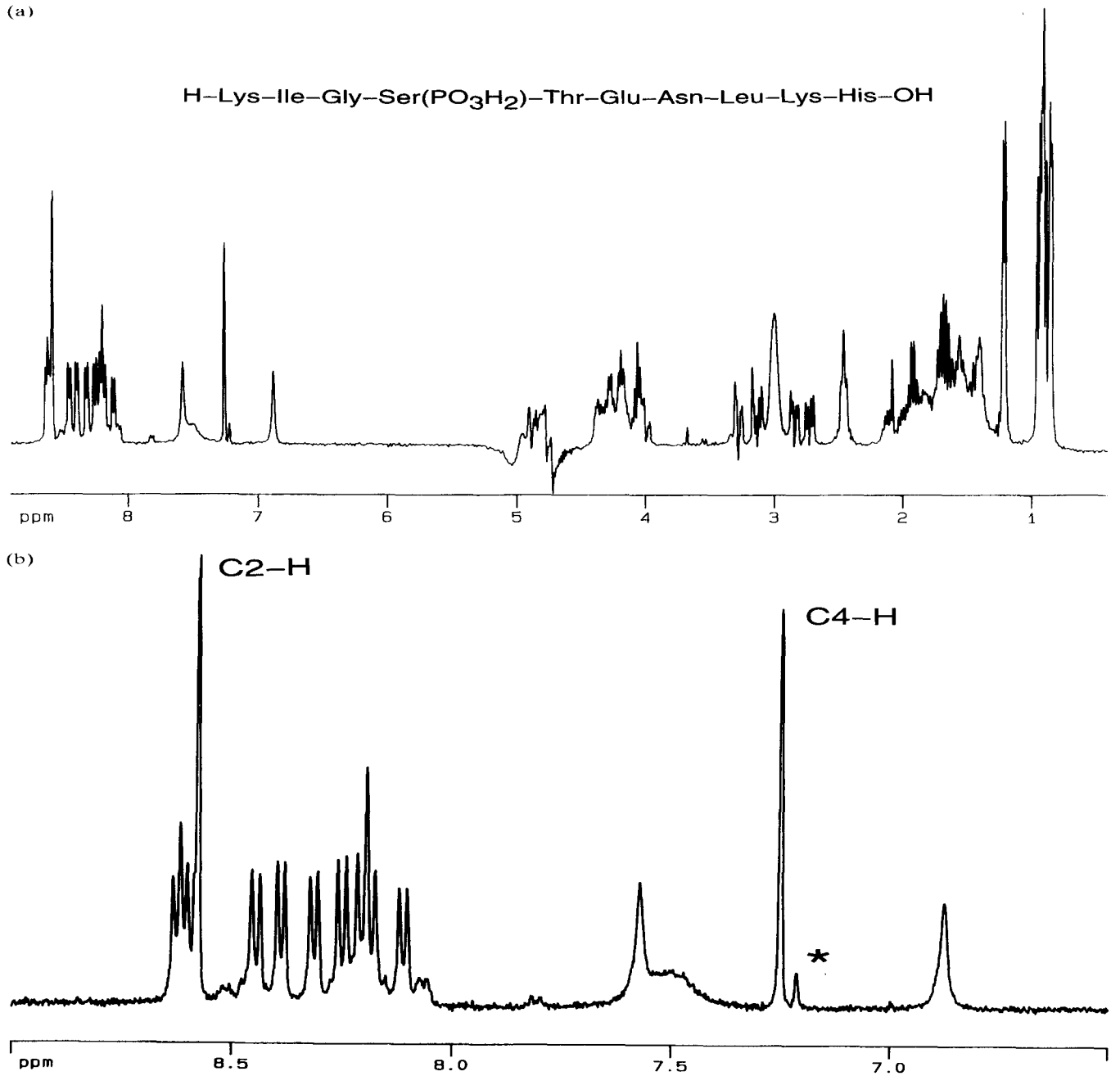


Figure 5. (a) ^{1}H NMR spectrum of phosphopeptide **1** in water. (b) Aromatic and amide spectral region of the ^{1}H NMR spectrum of phosphopeptide **1** in water . The aromatic resonances of the histidine are labelled. The asterisk indicates the C(4)-H His resonance of the minor component.

ation-dependent way, except for the antiserum raised against the phosphorylated peptide htau 197–205. The antiserum against this peptide recognized the phosphorylated peptide but not phosphorylated recombinant tau or native tau. This is probably due to a major conformational change in tau which occurs

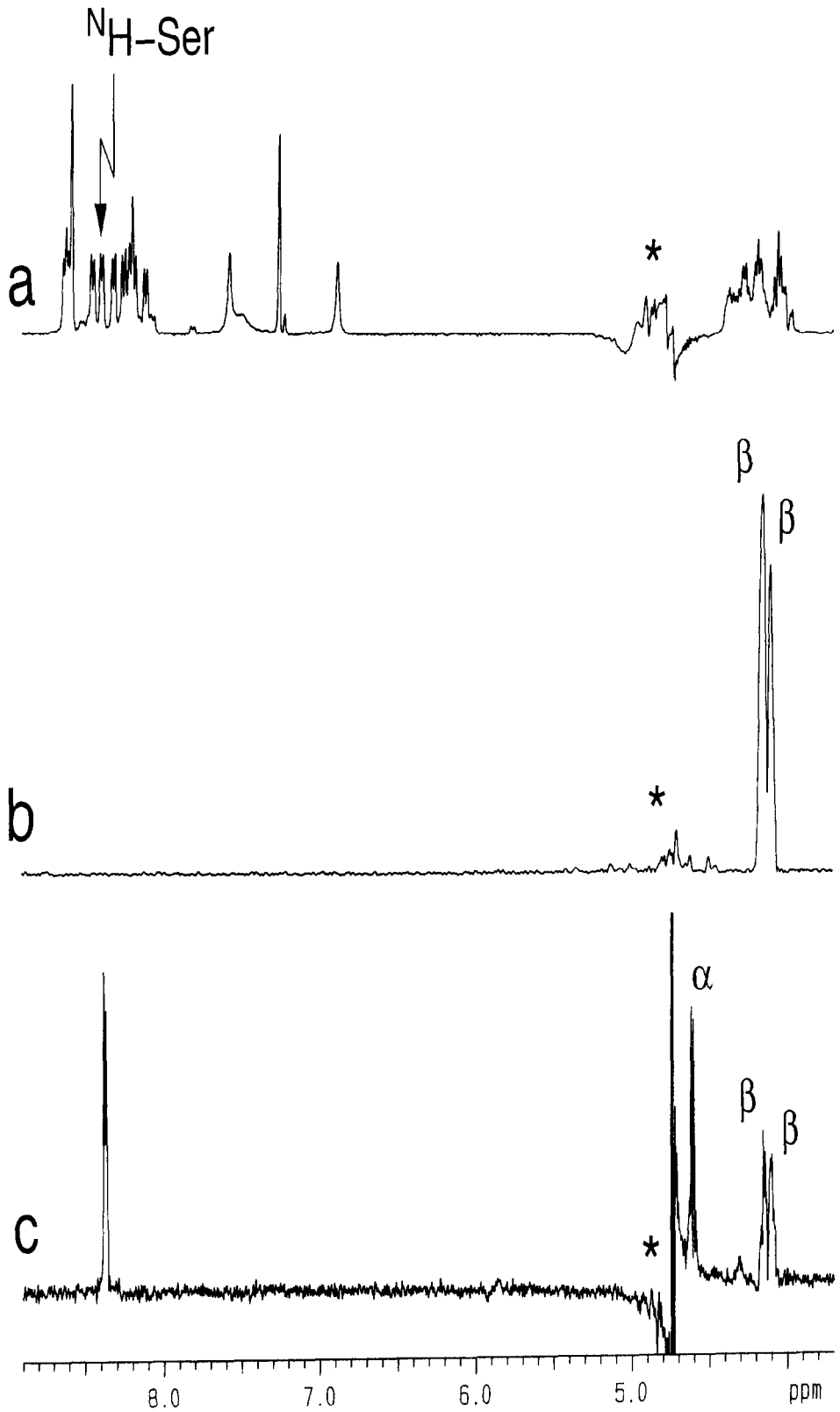


Figure 6. (a) ^{1}H NMR reference spectrum of **1** in water. (b) Row extracted from the 2-D HMBC ^{31}P ^{1}H correlated experiment. (c) One-dimensional PFG-selective TOCSY with selective excitation applied at the N—H resonance of the serine as indicated by the arrow in (a). The asterisks indicate the residual water signal.

around this site, when Ser-202 and Ser-199 are phosphorylated in native or in recombinant tau. The phosphorylation-dependent antisera[26] are well suited to study either tau in the brain during early development, where it is originally highly phosphorylated and gradually becomes dephosphorylated, or tau in Alzheimer's disease brain, where it is found in a highly phosphorylated, abnormal form.[27]

Discussion

In the conception of our method we had envisaged that it would be general, easy to perform and with the exception of the Alloc based residue consist essentially of standard Fmoc SPPS. In practice, after the establishment *N*-terminal Ser(PO_3H_2) on the resin, chain elongation via repeated standard Fmoc SPPS cycles was not a viable strategy. Thus, a fragment coupling was required to ensure a good yield of pure product. After we had developed our method the group of Wakamiya[28] reported a breakthrough method for synthesizing serine phosphopeptides which is based solely on standard Fmoc SPPS. Recently, the Roche group[29] has described the independent development of essentially the same method, which has been used for both serine and threonine phosphopeptide Fmoc synthesis. Very simply, the monobenzyl protected Fmoc-Ser[PO(OH)(OCH_2Ph)]—OH (**3**) was used as a building block in standard Fmoc-SPPS. The phosphodiester (**3**) is no longer sensitive to β-elimination under the conditions of Fmoc removal (20% piperidine–dimethylformamide) and this allowed SPPS of several serine phosphopeptides under standard Fmoc conditions. Generally, complications do not seem to arise from the use of the monoprotected phosphate. Furthermore, Wakamiya has compared the use of the free serine phosphate building block **4** in standard Fmoc SPPS to **3**.[28] Consistent with our results, he found the use of **4** to be accompanied by more side reactions.[30] Problems may be caused by activation of the free serine phosphate functionality in the coupling reactions or by phosphate dianion acting as an internal nucleophile under the basic conditions of Fmoc removal. Clarification of this issue will require further studies. Interestingly, such problems using **4** stand in marked contrast to the continued sequential chain extension with **3**. Indeed, the phosphodiester function such as that found in **3** has been described not to react with HBTU.[29] For purposes of comparison we have independently prepared **1** via Wakamiya's method. We found the method to be satisfactory and suitable for use in automated rapid Fmoc-synthesis using the HBTU coupling reagent.[31] The yield of **1** after purification was comparable to that obtained with our method.

In summary, a new combined Fmoc-Alloc general building block SPPS strategy has been successfully applied to the synthesis of the tau phosphopeptide **1**, which was inaccessible by the standard post synthetic phosphorylation strategy. At this point the standard Fmoc SPPS procedure using the now commercially available Fmoc-Ser[PO(OH)(OCH_2Ph)]—OH (**3**)[32] as a building block appears to be the most powerful method for synthesizing serine phosphopeptides. Other Boc- and Alloc-based SPPS building block methods have been reported which are limited to the specialist. There may be cases where the coupling of **3** onto a given *N*-terminus is problematic, while the coupling with fully protected **6** may be achieved and provide an alternative. Furthermore, the use of a building block such as **8** might also be useful in the former event. Cleavage of the phosphate allyl ester protecting group after incorporation would liberate the monobenzyl protected phosphodiester function on the resin. Fragment coupling strategies in conjunction with any of the methods will be useful when phosphopeptides of high purity are required since cleaner, more easily purified crude material is received.

Finally, our method may be useful in cases where a resin bound free phosphate group as found in **11** and **12** is required. Such a resin bound free serine phosphate has been crucial in the synthesis of peptide serine adenosine phosphates which are bisubstrate inhibitors of protein kinases.[33] These compounds were prepared by the coupling of adenosine phosphates to the resin-bound phosphopeptide, Boc-Leu-Arg(PMC)-Arg(PMC)-Ala-Ser(PO_3H_2)-Leu-Gly-Wang, in which the free serine phosphate was introduced by a postsynthetic phosphorylation-allyl phosphate deprotection protocol. For this class of compounds an attractive alternative building block method now exists for sequences which are not amenable to the postsynthetic phosphorylation methodology.

Experimental

General

Fmoc-Ser[PO(OH)(OCH_2Ph)]—OH (**3**) now available from Novabiochem, was prepared as described.[28] Other protected Fmoc amino acids and Fmoc-His(Trt)-Wang resin were purchased from Novabiochem, Switzerland. Fmoc-Gly-Sasrin and Boc-Lys(Boc)—OH were purchased from Bachem, Switzerland. Dimethylformamide (DMF), piperidine, methanol, methyl-*tert*-butylether (MTBE) and dichloromethane were purchased from E. Merck. Diisopropylcarbodiimide (DIC), 1-hydroxybenzotriazole (HOBt), trimethylsilyl azide, tetrabutylammonium fluoride trihydrate, ethanedithiol (EDT) and palladium tetrakistriphenylphosphine were purchased from Fluka AG. Keyhole limpet hemocyanin and Freunds adjuvant were purchased from SIGMA, nitrocellulose membranes from BioRad. Amino Acid analysis was performed at Sandoz. FABMS analysis was performed on a MAT 212 mass spectrometer using a thioglycerine matrix. NMR spectra were recorded on a Bruker AMX 400MHz instrument.

Manual peptide synthesis

Performed in the standard Fmoc mode[4] using a three-necked glass apparatus with a sintered glass underfitting to allow filtration under aspiration. Agitation of

the suspended resin was achieved with slow mechanical stirring. Fmoc-amino acids were preactivated with 1.0 equiv of DIC and HOBt in DMF and couplings were performed with 3.0 equiv for a standard period of 3 h. If necessary, the coupling was repeated (double coupling) until a negative Kaiser test[4] was obtained. Fmoc cleavages were performed with 20% piperidine-DMF for 10 min and DMF-MeOH-DMF (3X,3X,3X) resin washing was performed in between steps.

Analytical HPLC

Performed using a 5 μm C18-nucleosil 300 column (100 × 4 mm) from Macherey-Nagel (Switzerland) and a gradient 100A:0B, 40A:60B over 20 min; Flow = 1.5 mL min^{-1} (A = H_2O:H_3PO_4 100:0.1; B = CH_3CN:H_3PO_4 100:0.1). UV detection at 205 nm.

Semipreparative HPLC

Vydac 218TP1022 column (22 X 250 mm), C18 10 μm, water–acetonitrile 0.1% TFA gradient.

Alloc-Ser-OH:DCHA (8). Prepared in a standard fashion from Alloc-Ser—OH and recrystallized from methyl-*tert*-butyl ether, m.p. 148–150 °C, $[\alpha_D]^{20}$ +13.0 (*c* 1.6 CH_2Cl_2).

Alloc-Ser(P(O)(OAllyl)$_2$—OH (6). Larger scale preparation was carried out according to Lacombe et al.[13,14] To a solution of the Alloc-Ser-OH:DCHA (**8**; 3.54 g, 9.5 mmol) in dry dichloromethane (100 mL) under argon was added a solution of *tert*-butyldimethyl chlorosilane (1.57 g, 10.4 mmol) in CH_2Cl_2 (10 mL). The mixture was stirred for 30 min at room temperature, a solution of diallyl *N*,*N*-diisopropyl phosphoroamidite (4.64 g, 18.9 mmol) in CH_2Cl_2 (10 mL) and 1*H*-tetrazole (2.0 g, 28.4 mmol) was added and solution stirring was continued for 3 h at rt. The mixture was cooled to −40 °C and *tert*-butylhydroperoxide (6.3 mL, 3M in toluene, 18.9 mmol) was added stirring for 30 min at this temperature and 1.5 h at rt. After cooling the reaction to 0 °C, 100 mL of 10% $Na_2S_2O_5$ were slowly added the organic phase separated and washed once more with 100 mL with 10% $Na_2S_2O_5$. The solvent was evaporated and the residue dissolved in 50 mL of methyl *tert*-butylether followed by extraction with 10% citric acid (2 × 100 mL) and brine (1 × 100 mL). The organic phase was dried over Na_2SO_4 and concentrated to a dark yellow oil which was purified by flash chromatography eluting with a gradient CH_2Cl_2:MeOH 20:1 to CH_2Cl_2:MeOH:AcOH 10:1:0.1 This gave 1.4 g (42%) of Alloc-Ser(P(O)(OAllyl)$_2$—OH (**6**) as a light yellow oil: ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.45 (broad singlet, NH), 5.83–5.99 (m, 3H), 5.17–5.39 (m, 6H), 4.31–4.63 (m, 8H), 4.23–4.34 (m, 1H). FABMS MH^+ = 350. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 170.5, 155.8, 132.2, 131.7, 131.6, 118.8, 118.7, 117.7, 68.6, 67.4, 65.8, 53.9. $[\alpha_D]^{20}$ +25.2, (*c* 1.0 CH_2Cl_2).

Alloc-Ser(P(O)(OAllyl)$_2$:DCHA (6). In pure form the free acid **6** is sensitive to polymerization and as such is better stored in the cold in dilute solution. Alternatively, **6** can be converted to dicyclohexylamine (DCHA) salt in methyl-*tert*-butyl ether and recrystallized from ethyl acetate/methyl-*tert*-butyl ether. The DCHA salt of **6** is stable to storage at room temperature and can be converted to the free acid by extracting from 10% citric acid solution with methyl-*tert*-butyl ether; m.p. 99–104 °C. Anal. calcd for $C_{25}H_{43}N_2O_8P$: C, 56.59; H, 8.17; N, 5.28. Found: C, 56.52; H, 7.99; N, 5.24.

Boc-Lys(Boc)-Ile-Gly-OH. The protected fragment was prepared manually using standard Fmoc-SPPS chemistry on Sasrin resin starting from 0.5 mmol Fmoc-Gly-Sasrin (0.65 mmol g^{-1}). After the coupling of Boc-Lys(Boc)—OH the protected peptide was cleaved from the resin by stirring for 15 min with 12 mL 1% trifluoroacetic acid in dichloromethane three successive times. The combined filtrates were neutralized with pyridine and concentrated to a residue. The residue was dissolved in ethyl acetate and washed with 1% HCl. The organic phase was separated, dried over sodium sulfate and concentrated to 217mg of product as a white foam. This crude product, which was quite pure (ca. 80%) by HPLC analysis, was further purified by flash chromatography (silica gel, ethyl acetate:hexane 1:1) to give Boc-Lys(Boc)-Ile-Gly—OH in 60% yield. Analytical HPLC retention time 16.60min. FABMS: $M+Na^+$ = 539 (35), MH^+ = 517 (5), MH^+ -Boc = 417 (45), MH^+ −2Boc (80).

Phosphotau (259–268), H-Lys-Ile-Gly-Ser(PO_3H_2)-Thr-Glu-Asn-Leu-Lys-His—OH (1). H-Thr(But)-Glu(But)-Asn(Trt)-Leu-Lys(t-Boc)-His(Trt)-Wang (11) was prepared manually and the loading was determined by amino acid analysis to be 0.45 mmol/g. Resin **11** (600 mg, 0.23 mmol) was suspended in 4 mL DMF and a 2.5 mL DMF solution containing 3.0 equiv (0.69 mmol, 240 mg) of Alloc-Ser(P(O)(OAllyl)$_2$)—OH,which had been preactivated for 20 min with HOBt-DIC was added. After 3 h the resin was subjected to the standard washing cycles and in vacuo dried resin samples were analysed by Kaiser test and standard cleavage followed by HPLC and FABMS analysis. The remaining resin Alloc-Ser(P(O)(OAllyl)$_2$)-Thr(But)-Glu(But)-Asn(Trt)-Leu-Lys(t-Boc)-His(Trt)-Wang (**12**; 616 mg, ca 0.21 mmol) was suspended in 6 mL of dichloromethane and a premixed solution of trimethylsilylazide (0.65 mL, 5.0 mmol) and tetrabutylammonium trihydrate (590 mg, 1.9 mmol) in 6 mL of dichloromethane added under argon. Palladium tetrakistriphenylphosphine (70 mg, 0.61 mmol) was added and the mixture stirred for 30 min followed by filtration and standard resin washing. A portion of the resulting resin **13** (150 mg, ca 0.05 mmol) was suspended in 0.5 mL DMF and a 1.0 mL DMF solution containing 3.0 equiv Boc-Lys(Boc)-Ile-Gly—OH (78 mg, 0.15 mmol) preactivated for 40 min with DIC-HOBt was added. After 3 h the resin was washed and the coupling repeated in identical

fashion. Cleavage of the product from the support was performed by stirring the resin in 5 mL of TFA:EDT:H20 (90:5:5) over 3 h. The solvents were removed in vacuo and the residue washed with methyl-*tert*-butylether and dried to give 78 mg of crude product. A 58 mg sample was purified by semi-preparative HPLC to give 18.5 mg of lyophilized **1**. The average yield from three syntheses starting from Fmoc-His(Trt)-Wang was ca. 30%. The amino acid analysis of **1** was satisfactory and indicated a peptide content of ca. 80%. FABMS: $MH^+ = 1206$. Retention time for **1** = 4.58 min.

NMR methods

The one-dimensional ^1H NMR spectrum of 2.4 mg of **1** dissolved in 450 μL H_2O: 40 μL D_2O (pH3.3) shown in Figure 5(a) was recorded with the WATERGATE pulse sequence. The spectrum was recorded at 300 K on an AMX-400 Bruker spectrometer equipped with Z-gradient hardware. A total of eight scans were taken with a repetition time of 3.2 s. The length of the two 90° H_20 selective pulses and of the two sine-shaped pulsed field gradients were 4.1 ms and 1.8 ms, respectively. The data were multiplied by a cosine window function prior to Fourier transformation. For the expanded amide and aromatic region [Fig. 5(b)] 16 scans were recorded with a repetition time of 4.2 s. In addition a trim pulse of 600 μs length was applied before acquisition in order to destroy the antiphase dispersive component of the magnetization created during the WATERGATE scheme. Otherwise identical experimental parameters to those described for Figure 5(a) were used for the acquisition. The spectra shown in Figure 6 were recorded with an AMX-400 [Figs 6(a) and (b)] and with a DMX-500 Bruker spectrometer [Fig. 6(c)]. The experimental conditions for Figure 6(a) are the same as those described for Figure 5(a). The 2-D HMBC ^{31}P ^1H correlated spectrum in figure 6(b), was recorded with 32 scans for each of the 256 t_1 increments. The excitation heteronuclear multiple quantum period was 50 ms. The acquisition time and the repetition time were 0.18 and 1.5 s, respectively. The 1-D PFG selective TOCSY spectrum[34] shown in Figure 6(c) was recorded with a selective 180° Gaussian pulse of length 64 ms applied at the amide resonance indicated by the arrow in Figure 6(a). The length of the TOCSY step was 60 ms. A total of 800 scans were recorded with a repetition time of 1.9 s.

Rabbit polyclonal antisera

The htau phosphopeptides were dissolved in phosphate buffered saline (PBS) and coupled to keyhole limpet hemocyanin, suspended also in PBS, at a molar ratio of 20:1, employing either 0.2% glutardialdehyde or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide as a coupling reagent. The conjugates were dialysed against PBS. Aliquots equivalent to 100 μg of coupled peptide were emulsified in complete Freunds adjuvant and injected intradermally into female New Zealand rabbits. After 14 days, the immunization was repeated with conjugate emulsified in incomplete Freunds adjuvant. Thereafter, three to five booster injections were performed at monthly intervals and test bleeds were taken by the ear vein 7–10 days after each injection. Serial dilutions of the sera were tested against the peptides blotted on nitrocellulose membranes and against recombinant human tau $_{441}$ (clone htau 40, a generous gift of Dr M. Goedert, Cambridge). The antisera were also tested against phosphorylated tau. For this purpose, recombinant human tau was phosphorylated employing porcine or rat brain extract, as described.[35]

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