ChemComm

Cite this: *Chem. Commun.*, 2011, **47**, 10335–10337

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COMMUNICATION

A phosphoarginine containing peptide as an artificial SH2 ligand†

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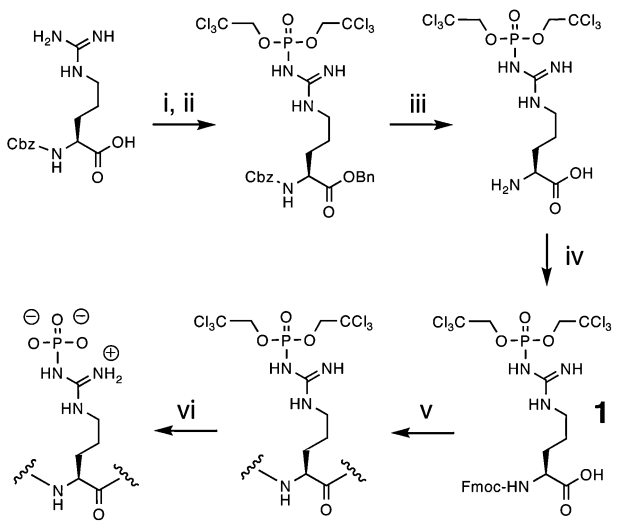
Received 5th June 2011, Accepted 3rd August 2011
DOI: 10.1039/c1cc13341a

We have developed a synthesis of phosphoarginine containing peptides using a bis(2,2,2-trichloroethyl) protected phosphoarginine derivative as building block. Binding studies and computer modelling demonstrate the ability of the SH2 domain from Src kinase to recognize a phosphoarginine-containing peptide in a phosphoryl group-dependent manner.

Reversible phosphorylation of protein side chains is a central mechanism in signal transduction and is therefore of key interest to further the understanding of cell signalling and the development of therapeutic interventions. Consequently, much attention has been focused on the complex network of kinases and phosphatases that control the phosphorylation state of thousands of serine, threonine and tyrosine side chains. In addition, phosphorylation of histidines, arginines and possibly lysines[1–3] may also contribute to phosphoproteomes, but their role in signal transduction is far less well understood. Recent accounts on phosphohistidine (pHis) containing proteins in mammalian cells,[2,4] the development of stable pHis analogs and pHis-specific antibodies[5,6] promise exciting insight into the physiological role of this posttranslational modification (PTM). Meanwhile, the discovery of a bacterial arginine kinase which phosphorylates a heat-shock regulator provided the first example of a signalling mechanism based on phosphoarginine (pArg).[7] This well characterized system complements previous reports on protein arginine kinase activity in several mammalian tissues and a number of pArg containing proteins.[8–12] However, more systematic prospecting of cellular pArg sites will be necessary to gauge their physiological role.

Synthetic access to specifically modified peptides has proven to be an important tool for the study of PTMs but current methods for arginine phosphorylation are either unspecific[13] or designed to prepare pArg as the free amino acid.[14,15] To overcome this limitation we here present the synthesis of peptides containing specific pArg residues by solid phase peptide synthesis (SPPS). For this building block approach the choice of protecting groups for the phosphoguanidinium moiety is crucial. Most widely used phosphate protecting groups require acidic conditions for their removal which would also cleave acid labile N–P bonds. Alternatively, trichlorethyl (Tc) protecting groups may be cleaved by hydrogenolysis at slightly alkaline pH after global deprotection of the peptide by acidic cleavage cocktails (Scheme 1).[16–19] If so, Tc protecting groups may prove suitable for the preparation of N-phosphorylated peptides.

To test this idea, we prepared bis(2,2,2-trichloroethyl) and Fmoc protected pArg (Scheme 1, **1**) in a four-step synthesis from Z-Arg-OH with an overall yield of 37% (see ESI†) and prepared three phosphopeptides using Fmoc-SPPS.[20] Peptides **2** and **3** (Scheme 2) represent the acetylated N- and C-termini of histone 3 which have been identified as substrates for arginine kinase activity in mammalian cells.[8] Peptide **4a** was designed as a potential ligand for the phosphotyrosine (pTyr) binding SH2 domain from Src kinase as detailed below. After peptide assembly, cleavage from the solid support with trifluoroacetic acid (TFA) yielded essentially pure bis(2,2,2-trichloroethyl) protected phosphoarginyl peptides, confirming that **1** is well suited for routine SPPS (Fig. S1, S4 and S7, ESI†).[20] Hydrogenolytic



Scheme 1 (i) BnBr, NMP, 5 h; (ii) bis(2,2,2-trichloroethyl)phosphoryloxychloride, NEt_3, CH_3CN, 7 h; (iii) H_2, Pd/C, AcOH/TFA; (iv) Fmoc-OSu, NEt3, CH_3CN, 1 h; (v) Fmoc-SPPS, 92.5% TFA; scavengers; (vi) H_2, Pd/C, pH 9.2.

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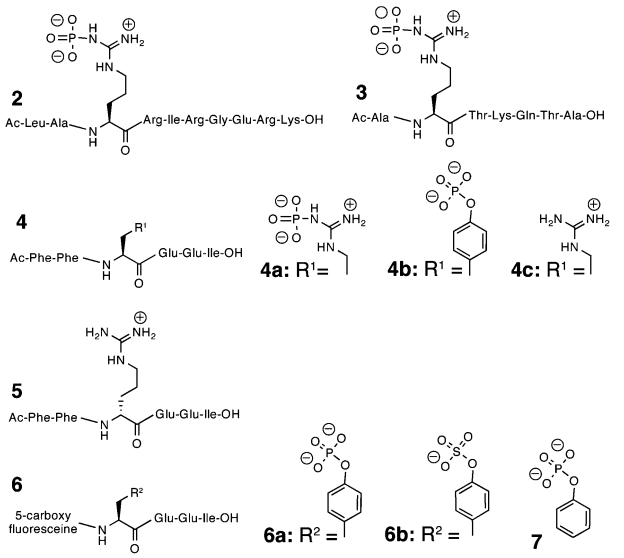
† Electronic supplementary information (ESI) available: Fig. S1–S14, Schemes S1–S4, detailed experimental procedures and characterization of **1** and synthetic intermediates thereof. See DOI: 10.1039/c1cc13341a



Scheme 2 pArg containing peptides an artificial SH2 ligands. **2** and **3** are phosphorylated derivatives of the N- and C- terminus of histone 3. **4** - **7** are potential SH2 ligands.

cleavage of the Tc groups in ammonia buffered aqueous ethanol at pH 9.2 released 69–75% **2**, **3**, and **4a** while causing less than 2% dephosphorylation (Fig. S2, S5 and S8, ESI†). Minor side products were removed by preparative HPLC using an acetonitrile gradient in alkaline aqueous buffer as a mobile phase (Fig. S3, S6 and S9, ESI†). We also enquired whether phosphopeptides generated by this methodology are stereochemically homogeneous. As an example, peptide **4a** was dephosphorylated in 50 mM HCl and compared with the two authentic diastereomers **4c** and **5** (Scheme 2). HPLC analysis of the three peptides showed that more than 99% of dephosphorylated **4a** co-eluted with **4c** suggesting that racemization is insignificant throughout the synthesis of **1** and that this building block is not prone to epimerization during Fmoc-SPPS (Fig. S10, ESI†).

As a first application of synthetic pArg containing peptides we studied a model protein–protein interaction mediated by this PTM. Modulation of protein complex stabilities is a major mechanism by which phosphorylated residues control protein activity as exemplified by SH2 domains, which recognize pTyr within short peptide motifs.[21] The human genome codes for more than one hundred SH2 domains which direct the cellular localization of a vast array of proteins by phosphate-dependent interactions. Because analogous pArg receptors are still unknown, we were interested in whether an SH2 domain could also recognize pArg in place of pTyr, and could ultimately serve as a blue print for designing phosphoarginine receptors.

The SH2 domain from Src kinase binds a phosphorylated peptide motif (pTyr-Glu-Glu-Ile) with a K_d of 180 nM while discriminating against the unphosphorylated version by 10^4-fold.[22] Peptides with phosphoserine (pSer)[22] or pHis[23] in place of pTyr are also poor ligands, demonstrating that the SH2 domain is highly sensitive to the length and geometry of the phosphate-bearing side chain. The side chain of pArg is longer than that of pSer and more flexible than that of pHis, and might therefore be a better match for the pTyr binding pocket.

To test this possibility we compared the affinities of the SH2 domain for peptides containing either pArg (**4a**) or arginine

Table 1 Binding affinity (K_d) of SH2 complexes with compounds **4**- **7** determined by a fluorescence polarization based displacement assay[a] or by direct fluorescence polarization[b]

Compound	K_d/μM
4a[a]	550 ± 150
4b[a]	0.13 ± 0.094
4c[a]	> 10 000
6a[b]	0.052 ± 0.012
6b[b]	2.9 ± 0.31
7[a]	270 ± 35

[a] Fluorescence polarization based displacement assay. [b] Fluorescence polarization.

(**4c**) in place of pTyr (**4b**). These measurements were performed using a fluorescence polarization based displacement assay[24–26] in which a complex between the SH2 domain and a fluorescein-labelled sensor peptide (**6a**) is titrated with the unlabeled analytes **4a–c**. We demonstrated that this assay is suitable for detecting low affinity interactions by measuring a K_d of 270 ± 35 μM (Fig. S11, ESI†) for the SH2 complex with phenyl phosphate (**7**) which is consistent with previous measurements using scintillation proximity binding.[27] We then determined the dissociation constants for the SH2 complexes with peptides **4b** (K_d = 130.0 ± 9.4 nM) and **4a** (K_d = 550 ± 150 μM) but we were unable to detect any interaction with **4c** below 10 mM (Table 1, Fig. S11–S12, ESI†). These data clearly establish phosphoryl-group dependent recognition of **4a** by the SH2 domain. However, exchanging pTyr with pArg results in a 4 × 10^3-fold lower SH2 affinity for the phosphopeptide.

To characterize the structural basis for this behaviour we built a model of the SH2 domain in a complex with a set of pArg rotamers based on commonly occurring Arg rotamers in the protein data bank (PDB).[28] We used the Rosetta software suite[29] to screen each of these rotamers by replacing the pTyr side chain in the crystal structure of the SH2 domain bound to pYEEI (PDB access code 1SHD).[30] Consistent with the observed recognition of peptide **4a** by the SH2 domain we found several pArg rotamers to project the phosphoryl group to a similar position and orientation as that of pTyr, allowing it to form equivalent interactions with ArgβB5 and ArgαA2 of the SH2 domain (Fig. 1, numbering according to ref. 22). The two salt bridges between these arginine side chains and the dianionic phosphate group on pTyr are crucial for high-affinity recognition by the src SH2 domain.[22] It is possible that the interaction with the monoanionic phosphoguanidinium side chain of pArg (Scheme 2) is weak predominantly because of a missing salt bridge. To quantify the effect of this missing charge we compared the affinities of the SH2 domain for peptide **6a** and the corresponding sulfotyrosine containing peptide **6b** (Scheme 2). The two compounds also differ by one negative charge but are otherwise strictly isosteric.[31] In a fluorescence polarization assay the sulfopeptide proved a 55-fold weaker SH2 ligand (Table 1, Fig. S13, ESI†) than **6a**, suggesting that charge difference alone is not the dominating factor in the 4 × 10^3-fold discrimination between pArg and pTyr.

Reinspection of our model points to additional challenges in pArg recognition. The SH2 domain recognizes pTyr by two cation-π interactions involving the side chains of ArgαA2 and

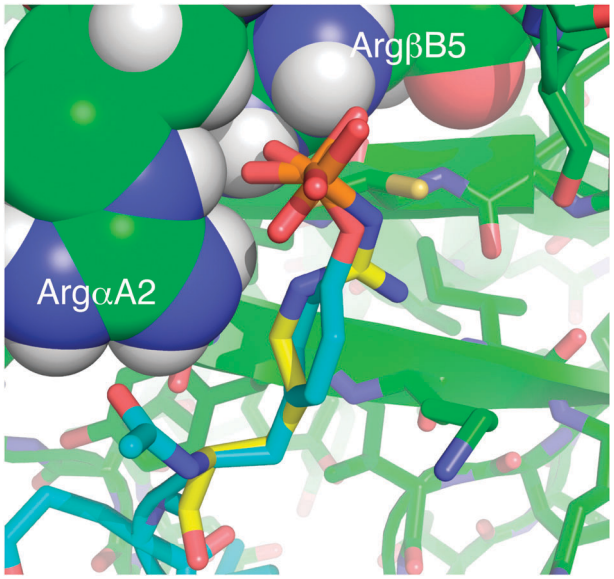


Fig. 1 Overlay of a pArg rotamer onto the Src SH2 domain in a complex with a pTyr containing peptide. ArgβB5 and ArgαA2 shown in space filling representation.

LysβD6 on either side of the aromatic ring (Fig. S14, ESI†). While these cationic sidechains form *stabilizing* interactions that contribute additional affinity for pTyr, we expect their contribution on the basic portion of the pArg side chain to instead be *destabilizing* due to direct electrostatic repulsion. The lack of a favourable electrostatic environment for pArg is further compounded by the greater loss of conformation entropy required for localization of the phosphoryl group in pArg relative to pTyr. Structural analysis of other SH2 domains may reveal those most suitable for pArg recognition, on the basis of the chemical character of the side chains forming the binding pocket.

In conclusion, we have presented a general method for the synthesis of pArg-containing peptides using **1** as a building block for SPPS coupled with selective deprotection of the phosphoryl group. Affinity measurements based on fluorescence polarization demonstrated phosphoryl group-dependent recognition of an arginine-phosphorylated peptide by the SH2 domain from Src kinase. This result in conjunction with computational modelling suggests that pTyr-binding proteins may serve as starting points for development of high-affinity reagents for the detection of proteins with phosphorylated arginine side chains.

We would like to thank R.S. Goody for financial support, H.D. Arndt and M.M. Müller for critical comments and S. Gentz for technical assistance. F.T. Hofmann was supported by the International Max Planck Research School in Chemical Biology. J.K. was supported by the Alfred P. Sloan Fellowship.

Notes and references

1 H. R. Matthews, *Pharmacol. Ther.*, 1995, **67**, 323–350.
2 P. V. Attwood, M. J. Piggott, X. L. Zu and P. G. Besant, *Amino Acids*, 2007, **32**, 145–156.
3 P. G. Besant, P. V. Attwood and M. J. Piggott, *Curr. Protein Pept. Sci.*, 2009, **10**, 536–550.
4 P. G. Besant and P. V. Attwood, *Mol. Cell. Biochem.*, 2009, **329**, 93–106.
5 T. E. McAllister, M. G. Nix and M. E. Webb, *Chem. Commun.*, 2011, **47**, 1297–1299.
6 J. M. Kee, B. Villani, L. R. Carpenter and T. W. Muir, *J. Am. Chem. Soc.*, 2010, **132**, 14327–14329.
7 J. Fuhrmann, A. Schmidt, S. Spiess, A. Lehner, K. Turgay, K. Mechtler, E. Charpentier and T. Clausen, *Science*, 2009, **324**, 1323–1327.
8 B. T. Wakim and G. D. Aswad, *J. Biol. Chem.*, 1994, **269**, 2722–2727.
9 B. T. Wakim, M. M. Picken and R. J. Delange, *Biochem. Biophys. Res. Commun.*, 1990, **171**, 84–90.
10 M. E. Wilson and R. A. Consigli, *Virology*, 1985, **143**, 516–525.
11 M. Sikorska and J. F. Whitfield, *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol.*, 1982, **703**, 171–179.
12 F. Levyfavatier, M. Delpech and J. Kruh, *Eur. J. Biochem.*, 1987, **166**, 617–621.
13 A. Kumon, H. Kodama, M. Kondo, F. Yokoi and H. Hiraishi, *J. Biochem.*, 1996, **119**, 719–724.
14 J. Bolte and G. M. Whitesides, *Bioorg. Chem.*, 1984, **12**, 170–175.
15 F. Cramer, A. Vollmar and E. Scheiffele, *Chem. Ber.*, 1962, **95**, 1670–1682.
16 Y. Liu, I. F. F. Lien, S. Ruttgaizer, P. Dove and S. D. Taylor, *Org. Lett.*, 2004, **6**, 209–212.
17 A. Paquet, *Int. J. Pept. Protein Res.*, 1992, **39**, 82–86.
18 A. Paquet, B. Blackwell, M. Johns and J. Nikiforuk, *J. Pept. Sci.*, 1997, **50**, 262–268.
19 A. Paquet and M. Johns, *Int. J. Pept. Protein Res.*, 1990, **36**, 97–103.
20 M. Ueki, K. Hashimoto and Y. Oka, *Peptide Sci.*, 2004, 169–172.
21 R. B. Jones, A. Gordus, J. A. Krall and G. MacBeath, *Nature*, 2006, **439**, 168–174.
22 J. M. Bradshaw, V. Mitaxov and G. Waksman, *J. Mol. Biol.*, 1999, **293**, 971–985.
23 L. Senderowicz, J. X. Wang, L. Y. Wang, S. Yoshizawa, W. M. Kavanaugh and C. W. Turck, *Biochemistry*, 1997, **36**, 10538–10544.
24 M. Hafner, E. Vianini, B. Albertoni, L. Marchetti, I. Grune, C. Gloeckner and M. Famulok, *Nat. Protoc.*, 2008, **3**, 579–587.
25 J. A. Cruz-Aguado and G. Penner, *Anal. Chem.*, 2008, **80**, 8853–8855.
26 T. J. Burke, K. R. Loniello, J. A. Beebe and K. M. Ervin, *Comb. Chem. High Throughput Screening*, 2003, **6**, 183–194.
27 D. Lesuisse, G. Lange, P. Deprez, D. Benard, B. Schoot, G. Delettre, J. P. Marquette, P. Broto, V. Jean-Baptiste, P. Bichet, E. Sarubbi and E. Mandine, *J. Med. Chem.*, 2002, **45**, 2379–2387.
28 R. L. Dunbrack and F. E. Cohen, *Protein Sci.*, 1997, **6**, 1661–1681.
29 A. Leaver-Fay, M. Tyka, S. M. Lewis, O. F. Lange, J. Thompson, R. Jacak, K. Kaufman, P. D. Renfrew, C. A. Smith, W. Sheffler, I. W. Davis, S. Cooper, A. Treuille, D. J. Mandell, F. Richter, Y. E. Ban, S. J. Fleishman, J. E. Corn, D. E. Kim, S. Lyskov, M. Berrondo, S. Mentzer, Z. Popović, J. J. Havranek, J. Karanicolas, R. Das, J. Meiler, T. Kortemme, J. J. Gray, B. Kuhlman, D. Baker and P. Bradley, *Methods Enzymol.*, 2011, **487**, 545–574.
30 T. Gilmer, M. Rodriquez, S. Jordan, R. Crosby, K. Alligood, M. Green, M. Kimery, C. Wagner, D. Kinder, P. Charifson, A. M. Hassell, D. Willard, M. Luther, D. Rusnak, D. D. Sternbach, M. Mehrotra, M. Peel, L. Shampine, R. Davis, J. Robbins, I. R. Patel, D. Kassel, W. Burkhart, M. Moyer, T. Bradshaw and J. Berman, *J. Biol. Chem.*, 1994, **269**, 31711–31719.
31 I. Catrina, P. J. O'Brien, J. Purcell, I. Nikolic-Hughes, J. G. Zalatan, A. C. Hengge and D. Herschlag, *J. Am. Chem. Soc.*, 2007, **129**, 5760–5765.