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Solid-phase synthesis of Stat3 inhibitors incorporating *O*-carbamoylserine and *O*-carbamoylthreonine as glutamine mimics

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Abstract—*O*-Carbamoylserine and *O*-carbamoylthreonine are glutamine analogues that were incorporated into a Stat3 inhibitory peptide to probe the requirements of Gln at the pY+3 position. Fmoc-Ser-NHBn and Fmoc-Thr-NHBn were converted to nitrophenyl carbonates and were attached to Rink resin via a side-chain carbamate linkage. After assembly of the peptide, acid treatment resulted in *O*-carbamoylserine and *O*-carbamoylthreonine-containing peptides. The order of affinity for Stat3 was Gln > Ser($CONH_2$) ≫ Thr($CONH_2$) suggesting a relatively tight binding pocket for the side chain of glutamine.

Stat3 (signal transducer and activator of transcription 3) is a cytosolic transcription factor that transmits signals directly from cell-surface receptors to the nucleus. On cytokine or growth factor binding, Stat3 is recruited to phosphotyrosine on the receptors via its SH2 domain. It becomes phosphorylated on Tyr705, dimerizes, translocates to the nucleus, and initiates transcription of survival proteins such as Bcl-$_{XL}$, and cell-cycling proteins such as cyclin D1. Stat3 is constitutively activated in a number of tumors and studies with antisense and decoy oligonucleotides result in apoptosis of tumor cells. Thus, this protein is a target for anticancer drug design (Reviewed in Refs. 1–5).

Targeting the SH2 domain would uncouple Stat3 from the growth and survival signaling pathways and is a reasonable approach for the development of anticancer chemotherapeutic agents. The consensus recognition sequence for this protein, pTyr-Xxx-Xxx-Gln, shows that Stat3 requires a glutamine at the pY+3 position.[6–8] Glutamine analogues that escape recognition by proteases and glutaminases would enhance activity of Stat3 inhibitors in physiological environments. *O*-Carbamoylserine (Ser($CONH_2$)) is a glutamine mimic in which the γ-methylene group is replaced with an oxygen atom (Fig. 1). *O*-Carbamoylthreonine (Thr($CONH_2$)) is a β-methyl-substituted analogue. *O*-Carbamoylserine was first reported in 1956 as an antibiotic[9] and bacterial growth inhibition was reversed by adding Glu or Gln to the culture media demonstrating glutamine mimicry properties. Ser($CONH_2$) was also used as a Gln mimic in bradykinin analogues[10,11] and has activity as a glutaminase inhibitor.[12] We therefore investigated its utility as a Gln mimic in phosphopeptide inhibitors of Stat3.

Solid-phase synthesis of peptoid polymers possessing carbamate resin linkage and urethane peptide bond replacements was reported by P. Schultz and colleagues.[13–15] Fmoc-amino acids were reduced to the

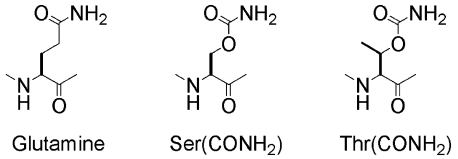

Figure 1. Structure of glutamine and its carbamate mimics.

Keywords: Carbamoylserine; Carbamoylthreonine; Stat3; Signal transducer and activator of transcription 3; Solid-phase peptide synthesis; Rink resin; Phosphopeptide; Peptidomimetic.
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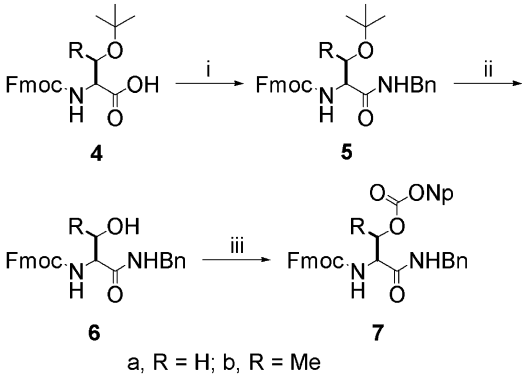
corresponding alcohols which were activated as stable mixed carbonates with 4-nitrophenyl chloroformate. Coupling of these to Rink resin ultimately led to C-terminal carbamate. Alsina et al.[16] reported the use of in situ activation of an Fmoc-amino-alcohol with disuccinimidyl carbonate and attaching this to aminomethyl resin to prepare resin-bound C-terminal carbamate. After peptide chain extension, acid cleavage gave the C-terminal carbamate. Fernandez-Forner et al.[17] extended this technique to synthesize *O*,*N*-substituted carbamates. There are no reports on the solid-phase synthesis of peptides or peptidomimetics incorporating Ser($CONH_2$) or Thr($CONH_2$).

In a screen of phosphopeptides targeting the SH2 domain of Stat3, we found that Ac-pTyr-Leu-Pro-Gln-Thr-Val-NH_2 (**1**) was a high-affinity inhibitor.[18] Structure–activity studies revealed that the C-terminal Thr-Val-NH_2 dipeptide could be substituted with benzyl amide with minimal loss of activity.[19] In fluorescence polarization assays Ac-pTyr-Leu-Pro-Gln-NHBn (**2**) had an IC_{50} of 409 nM vs 290 nM for peptide **1** (Table 1).[19] Replacement of the N-terminal Ac-pTyr unit with 4-phosphorylcinnamate enhanced activity giving an IC_{50} of 135 nM for pCin-Leu-Pro-Gln-NHBn (**3**).[20] We sought to convert the glutamine residue of peptide **3** to *O*-carbamoylserine and *O*-carbamoylthreonine. In this communication, we report the solid-phase synthesis of phosphopeptides containing carbamoylserine and carbamoylthreonine and their evaluation as Stat3 inhibitors.

Fmoc-protected *O*-carbamoylserine and threonine building blocks were prepared as activated *O*-nitrophenyl carbonates (Scheme 1). Fmoc-Ser(*t*-Bu)-OH (**4a**) was coupled with benzylamine using water-soluble carbodiimide and the *tert*-butyl ester removed with TFA to give Fmoc-Ser-NHBn, **6a**. Treatment with 1.1 equiv of 4-nitrophenylchloroformate and pyridine gave the mixed carbonate ester, Fmoc-Ser(CO_2Np)-NHBn, **7a**, in high yield. The carbonate was stable to silica-gel chromatography which was used to remove excess chloroformate.[21]

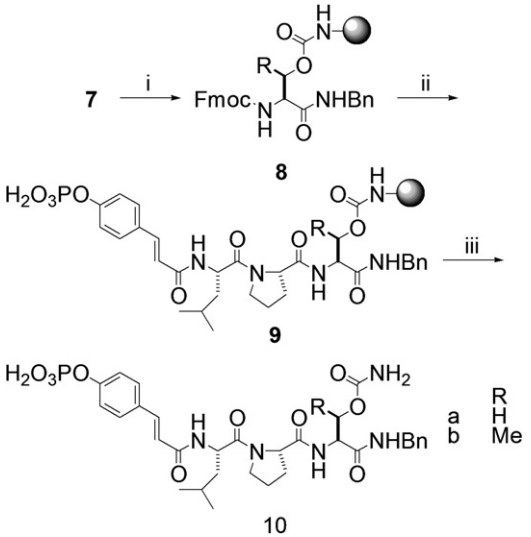
The same set of reactions were used to prepare Fmoc-Thr(CO_2Np)-NHBn, **7b**.[21] It should be noted that the intermediate Fmoc-Thr-NHBn, **6b**, is a rather insoluble compound and that coupling with 4-nitrophenylchloroformate required extended reaction times.

Three equivalents of the nitrophenyl carbonates, **7a** and **7b**, were attached to Rink resin via the side chain in the presence of 4 equiv of DIEA in 1:1 DMF/CH_2Cl_2 (Scheme 2). The release of 4-nitrophenol was evident by the yellow color of the supernatant as the carbamate linkages were formed. Couplings were complete in 1–3 h, as judged by ninhydrin tests. Peptide synthesis was carried out as follows. Fmoc groups were removed by treating the resin 2× with 20% piperidine in DMF for 5 min each. Resin washes were accomplished by five treatments with 1:1 DMF/CH_2Cl_2. Fmoc-Pro-OH and Fmoc-Leu-OH were coupled by adding 3 equiv each of the amino acid, 1-hydroxybenzotriazole (HOBt), and diisopropylcarbodiimide until ninhydrin tests were negative. 4-Phosphorylcinnamic acid, prepared as described by Szardenings et al.,[22] was coupled in 3-fold excess with 3 equiv each of PyBOP, HOBt, and 6 equiv of DIEA. Peptides were cleaved from the supports with TFA/triisopropylsilane/H_2O (95:2.5:2.5), precipitated in Et_2O, and were purified to >98% purity by reverse-phase HPLC using gradients of acetonitrile in 0.01 M NH_4OAc to give peptides **10a** and **10b**.[23]



Scheme 1. Reagents: (i) $BnNH_2$, EDC, CH_2Cl_2; (ii) TFA; (iii) $ClCO_2C_6H_4NO_2$, pyridine.

Peptides **10a** and **10b** were assayed for their ability to bind to Stat3 by competing with Fl-Ala-pTyr-Leu-Pro-Gln-Thr-Val-NH_2 (Fl = 5-carboxyfluorescein) in a fluo-



Scheme 2. Reagents: (i) Rink resin, DIEA, DMF/CH_2Cl_2; (ii) peptide synthesis; (iii) TFA/TIS/H_2O (95:2.5:2.5).

Table 1. Inhibition of Stat3

Compound	Sequence	IC_{50} (nM)[a]
1	Ac-pTyr-Leu-Pro-Gln-Thr-Val-NH_2	290 ± 63[b]
2	Ac-pTyr-Leu-Pro-Gln-NHBn	409 ± 15[b]
3	pCin-Leu-Pro-Gln-NHBn	135 ± 8
10a	pCin-Leu-Pro-Ser($CONH_2$)-NHBn	379 ± 49
10b	pCin-Leu-Pro-Thr($CONH_2$)-NHBn	850 ± 85

[a] Values are means of three experiments ± standard deviation.
[b] From Ref. 19.

rescence polarization assay.[19] Peptide **10a**, containing Ser($CONH_2$), exhibited an IC_{50} of 379 nM, which was approximately 3-fold less active than peptide **3**. Interestingly, peptide **10b** was 6-fold less active.

Previously, we showed that replacing the side-chain amide protons of glutamine with one or two methyl groups severely impaired binding of phosphopeptides to Stat3.[19] Furthermore, replacing Gln with isosteric methionine sulfoxide caused a 20-fold reduction in affinity. Taken together with the observation that Gln at pY+3 is a specificity determinant for binding to Stat3, these results suggest that the side-chain amide protons form important hydrogen bonds with groups on the protein. It is unclear whether the loss in affinity of **10a** is due to reduced hydrogen bonding, slightly altered geometry of the $OC(O)NH_2$ group relative to the $CH_2C(O)NH_2$ of **3**, increased energy of de-solvation of the carbamate group, or other factors. However, the 6-fold loss in affinity of **10b** suggests that the side chain of Gln resides in a tight pocket or cleft, and that the β-methyl group of Thr($CONH_2$) results in steric clash with this site.

In summary, we have synthesized peptides containing Ser($CONH_2$) and Thr($CONH_2$) using solid-phase techniques. As glutamine analogues, these amino acids reduce affinity of a peptide for the SH2 domain of Stat3, which requires Gln at the pY+3 residue. There are currently no X-ray crystallographic or NMR structures of Stat3 complexed with Y(p)XXQ peptides that would reveal phosphopeptide–protein interactions. Thus, the reduced activity of the threonine analogue provides insight into the binding pocket for Gln.

Acknowledgments

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20. **3** ESI-MS calcd, 671.27; found, 671.35. Analytical C18 HPLC T_R (a) 18.02 min 0–40% ACN/30 min (both solvents with 0.1% TFA) (b) 15.28 min 0–40% ACN in 0.01 M NH_4OAc.
21. Compound **7a** white solid mp 167–169 °C. ^1H NMR (300 MHz) $CHCl_3\delta$ 3.6–3.67 (m, 2H), 4.19–4.24 (m, 2H), 4.34–4.45 (m, 2H), 4.54 (s, 2H), 5.24 (br s, 1H), 7.25–7.42 (m, 11H), 7.58 (d, $J = 7.5$ Hz, 2H), 7.76 (d, $J = 7.5$ Hz, 2H), 8.22 (d, $J = 7.5$ Hz, 2H). ESI-MS calcd, 581.18; found, 582.30. Compound **7b** white solid mp 193–195 °C. ^1H NMR (300 MHz) $CHCl_3\delta$ 1.37 (d, $J = 6.6$ Hz, 3H), 4.22 (t, $J = 6.3$ Hz, 1H), 4.42–4.6 (m, 5H), 5.48 (m, 1H), 5.61 (d, $J = 8.1$ Hz, 1H), 6.48 (br s, 1H), 7.28–7.45 (m, 12H), 7.59 (d, $J = 7.2$ Hz, 2H), 7.78 (d, $J = 7.5$ Hz, 2H), 8.26 (d, $J = 9.3$ Hz, 2H). ESI-MS calcd, 595.20; found, 596.40.
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23. Compound **10a** ESI-MS calcd, 673.25; found, 673.32. Analytical C18 HPLC T_R (a) 15.58 min, 0–40% ACN/30 min (both solvents with 0.1% TFA) (b) 13.85 min, 0–40% ACN in 0.01 M NH_4OAc. Compound **10b** ESI-MS calcd, 687.27; found, 687.28. Analytical C18 HPLC T_R (a) 19.07 min, 0–40% ACN/30 min (both solvents containing 0.1% TFA) (b) 13.85 min, 0–40% ACN in 0.01 M NH_4OAc.