COMMUNICATION

Chemoenzymatic synthesis of a biotin-labeled glycophosphononapeptide of the c-Myc oncoprotein

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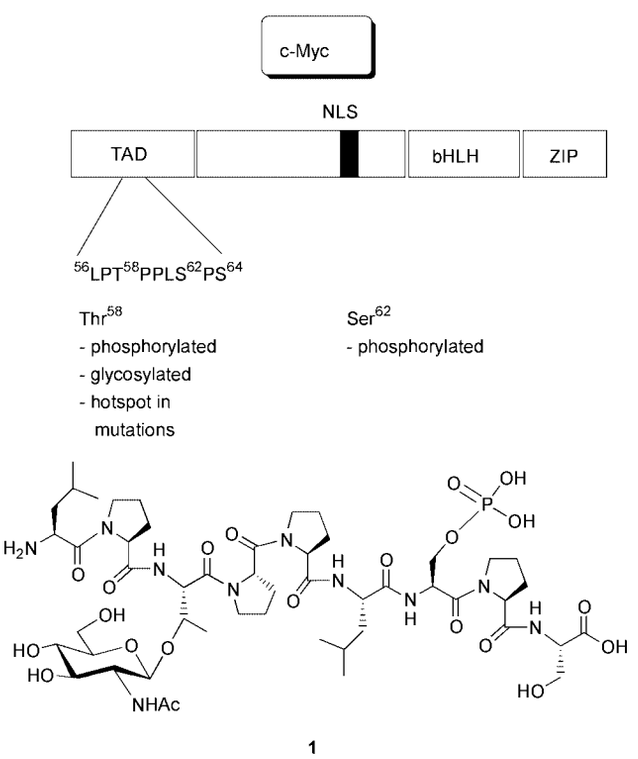
Received (in Cambridge, UK) 14th June 2000, Accepted 22nd June, 2000
Published on the Web 10th July 2000

Glycophosphopeptides that represent characteristic partial sequences of the posttranslationally modified transcriptional activation domain of the c-Myc oncoprotein can be built up efficiently by a combination of enzymatic and classical chemical techniques.

c-Myc is a nuclear transcription factor that plays a critical role in the regulation of gene transcription in cell proliferation, cell differentiation and programmed cell death in normal and neoplastic cells.[1,2] For neoplastic transformation,[3a] inhibition of cellular differentiation,[3b] and induction of apoptosis[3c] mediated by c-Myc, the *N*-terminal transcriptional activation domain (TAD; see Scheme 1) of the protein is required. Phosphorylation at Thr-58 and/or Ser-62 in the TAD of c-Myc has been suggested to modulate the transactivation[4a] and cellular transformation by Myc.[4b] Recently it was also shown that Thr-58 is the major site of covalent attachment of *N*-acetylglucosamine to the c-Myc oncoprotein[5] (Scheme 1). Furthermore, Thr-58 is the most important 'hotspot' in mutations in c-Myc found in different types of tumours. This mutation is associated with increased tumourigenicity, suggesting that posttranslational modification at Thr-58 leads to negative regulation of growth and neoplastic phenotype.



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Scheme 1 Structure of the c-Myc protein. TAD = transcriptional activation domain; NLS = nuclear localization sequence; bHLH = basic helix-loop-helix domain; ZIP = leucine zipper domain.

Covalent modification of serine and threonine residues by phosphate and *O*-GlcNAc are highly dynamic processes which often are reciprocal to each other.[6] Therefore, currently it is unclear which of these modifications at Thr-58 of c-Myc are beneficial for preventing the oncogenic properties of this protein. It has, however, been suggested that the form of c-Myc that interacts with tumour suppressors like the retinoblastoma protein (Rb) or the p53 protein is the *O*-GlcNAc-modified form.[6]

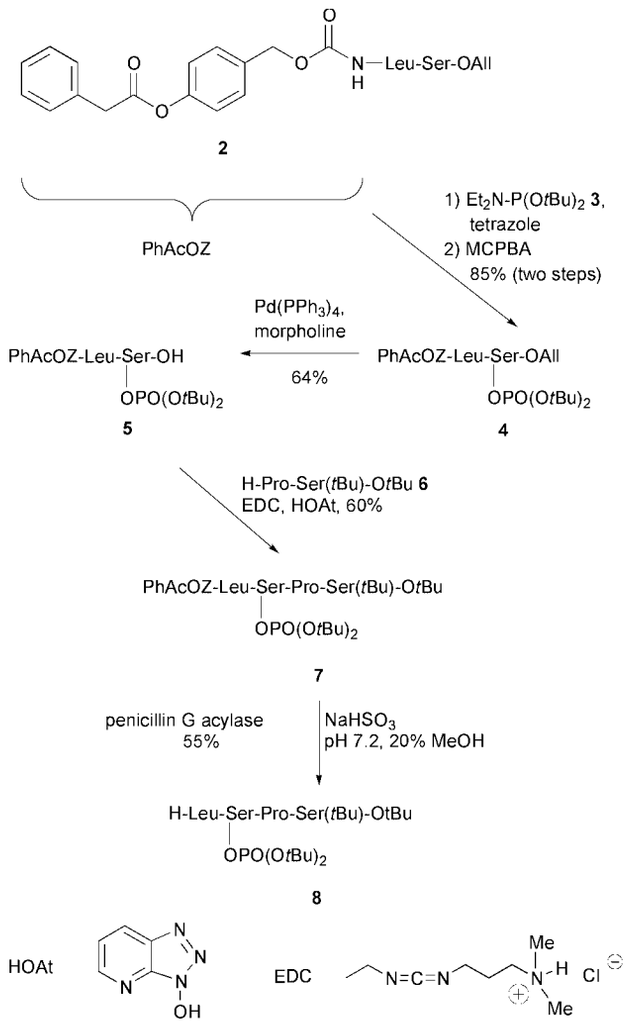
In order to study the biological phenomena associated with covalent modification of c-Myc, characteristic phosphorylated and glycosylated peptides may be useful reagents. We now report that c-Myc glycophosphopeptides can be built up efficiently by combination of enzymatic and classical chemical techniques.

The nonapeptide **1** was chosen as biologically relevant target compound that represents the correctly glycosylated and phosphorylated partial sequence present in the form of Myc that interacts with the Rb tumour suppressor (*vide supra*).

In the construction of the peptide conjugate **1**, the pronounced acid- and base-lability of glycosylated and phosphorylated peptides had to be taken into account. Thus both glyco- and phosphopeptides undergo a facile β-elimination of the entire carbohydrate and phosphate even under weakly basic conditions,[7,8] and at acidic pH an anomerization or rupture of *O*-glycosidic bonds can occur.[8] Therefore, a set of blocking functions is required that can be removed under the mildest (preferably neutral) conditions but are orthogonally stable. To meet these demands we employed the enzyme-labile *p*-phenylacetoxybenzyloxycarbonyl group as the temporary *N*-terminal protecting group,[9] and the Pd^0-sensitive allyl ester[10] as the *C*-terminal blocking function. The carbohydrate was *O*-acetylated to guarantee enhanced acid-stability of the *O*-glycosidic bond,[9,11] and the phosphate was masked with *tert*-butyl groups.[12]

PhAcOZ-protected dipeptide allyl ester **2**[13] was *O*-phosphorylated by treatment with phosphitylating reagent **3** and subsequent oxidation (Scheme 2). The phosphopeptide **4** thereby obtained then was selectively deprotected at the *C*-terminus by Pd^0-mediated transfer of the allyl group to morpholine as the accepting nucleophile. Coupling of the resulting carboxylic acid **5** with the *N*-terminally unmasked dipeptide *tert*-butyl ester **6** delivered the fully protected phosphotetrapeptide **7**. From this intermediate the enzyme-labile PhAcOZ urethane was cleaved off under neutral conditions. In the enzymatic transformation the biocatalyst recognizes and cleaves the phenylacetate incorporated into the blocking group. Thus a phenolate is liberated which undergoes a spontaneous non-enzymatic fragmentation to give a quinomethane, CO_2 and the desired *N*-terminally deprotected peptide. The quinomethane is

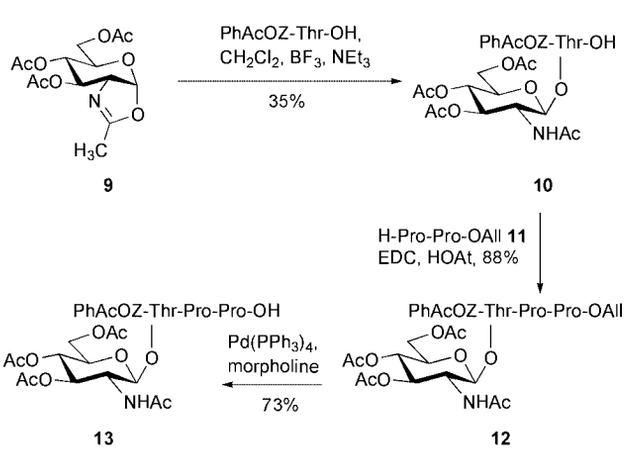
DOI: 10.1039/b004822o



Scheme 2 Synthesis of the selectively deprotected phosphopeptide fragment **8**.

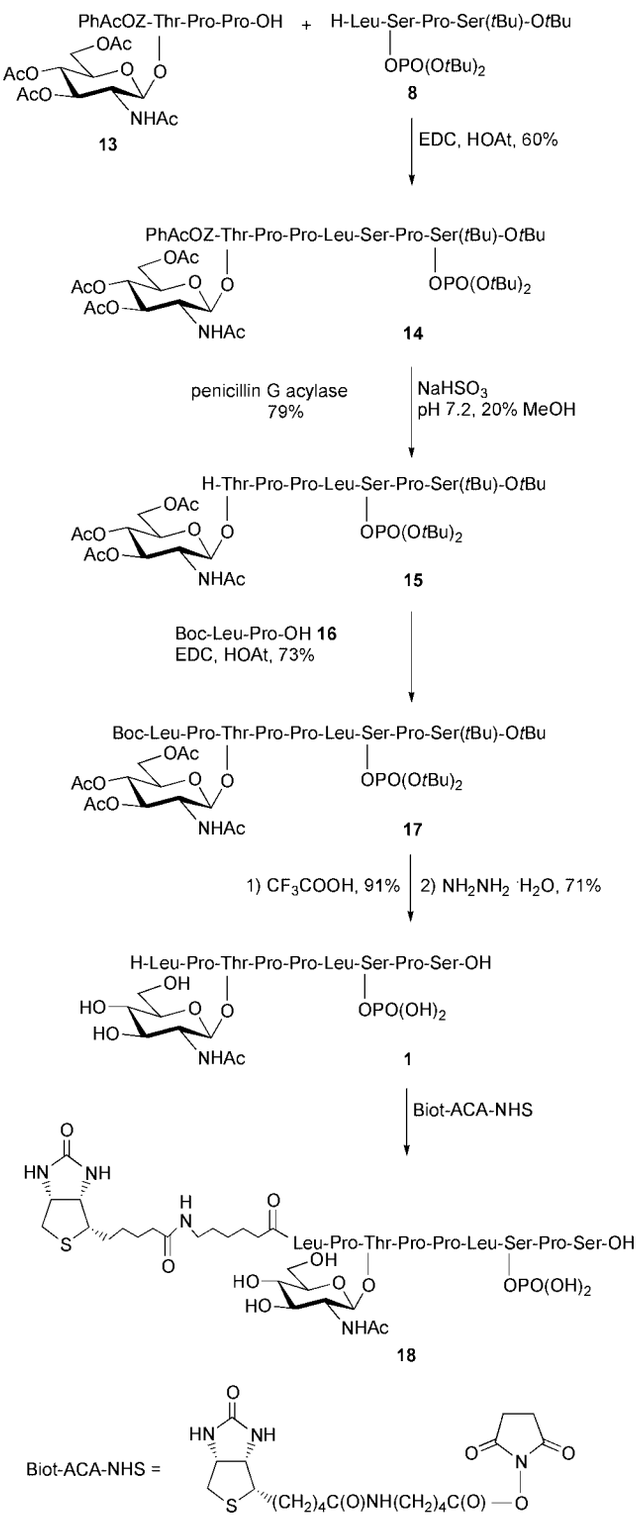
readily trapped by water or $NaHSO_3$ as nucleophile. In both the Pd^0-mediated removal of the allyl ester and the enzymatic cleavage of the PhAcOZ urethane no undesired side reaction was observed. The reaction conditions are so mild that β-elimination of the phosphate does not occur. Also, the substrate specificity of the biocatalyst guarantees that exclusively the phenylacetic acid ester is cleaved and that the *C*-terminal ester group, the phosphate and the peptide bonds are left intact.

Next, the PhAcOZ-protected threonine derivative **10** was synthesized from oxazoline **9**[14] and PhAcOZ-Thr and then condensed with peptide allyl ester **11** to give the completely protected glycotripeptide **12** in high yield (Scheme 3). From **12**, the *C*-terminal allyl ester was removed by Pd^0-mediated allyl transfer to morpholine to give carboxylic acid **13**.



Scheme 3 Synthesis of the glycopeptide fragment of **13**.

The selectively deprotected glycotripeptide **13** and phosphotetrapeptide **8** were then coupled to give the complex glycophosphopeptide **14** in high yield (Scheme 4). Peptide conjugate **14** embodies an acid- and base-labile carbohydrate and a base-labile phosphate group. Nevertheless the *N*-terminal PhAcOZ group was removed from this sensitive multifunctional compound without any undesired side reaction by penicillin G acylase initiated fragmentation of the urethane. Once more the substrate specificity of the biocatalyst guarantees that all other functional groups present are left intact. The reaction conditions again are so mild that neither β-elimination nor anomerization occur. By means of this enzymatic transformation the desired selectively deprotected glycophosphoheptapeptide **15** was obtained in high yield (Scheme 4). After



Scheme 4 Synthesis of the c-Myc glycophosphononapeptide **1** and the biotin labeled analogue **18**.

further elongation of the peptide chain by Boc-protected dipeptide **16**,[13] all protecting groups were removed from the glycosylated and phosphorylated nonapeptide **17**. To this end, first all acid-labile *tert*-butyl groups were cleaved off in one step by means of trifluoroacetic acid and the *O*-acetates were saponified selectively by treatment with hydrazine hydrate in methanol to give target compound **1** (Scheme 4).

Finally, the glycophosphononapeptide **1** was equipped with a biotin label by selective *N*-acylation with biotinylaminocaproic acid *N*-hydroxysuccinimide (Biotin-ACA-NHS; Scheme 4). The biotinylated peptide conjugate **18** obtained in this way may serve as an efficient molecular probe. The biotin label can be traced by means of the protein streptavidin which is available in fluorescently labeled form or modified with colloidal gold thus allowing the study of a glycosylated and phosphorylated model protein *e.g.* by fluorescence microscopy and electron microscopy in eukaryotic cells.[15]

In conclusion we have devised a method for the synthesis of glycosylated and phosphorylated c-Myc peptides by combination of enzymatic and classical chemical transformations. By means of this methodology biologically relevant labeled peptide conjugates can be built up which may open up new avenues of research in biology and bioorganic chemistry.[16] In particular, they should serve to unravel the chemical biology of the c-Myc protein and the importance of its posttranslational modification in molecular detail.

Experimental

Pd(0)-Mediated cleavage of the allyl ester from phosphopeptide 5 and glycopeptide 13

To a solution of the peptide allyl ester (0.5 mmol) and 2 mol% of $(Ph_3P)_4Pd$ in 20 mL of THF under an argon atmosphere was added dropwise a solution of morpholine (53 μL, 0.6 mmol) in 5 mL of THF. The mixture was stirred for 30 min, the solvent was removed under reduced pressure and the residue purified by flash chromatography (silica gel, CH_2Cl_2–EtOH mixtures).

Enzymatic removal of the PhAcOZ group from phosphopeptide 7 and glycophosphopeptide 14

To a solution of the respective PhAcOZ-protected peptide (0.02 mmol) in 8 mL of MeOH, 32 mL of Na_2HPO_4 buffer was added, which contained 50 mM $NaHSO_3$ and was adjusted to pH 7.2 by addition of 0.02 M NaOH. This solution was treated with penicillin G acylase (160 units) for 4–12 h at room temperature. The immobilized enzyme was filtered, and the MeOH was removed under reduced pressure. After lyophilisation, the crude residue was purified by flash chromatography (silica gel, $CHCl_3$–MeOH or ethyl acetate–EtOH mixtures).

Acknowledgements

This research was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

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13 PhAcOZ-protected dipeptide 2 was built up in 84% yield, by coupling of PhAcOZ-Leu-OH and H-Ser-OAll with EEDQ as coupling reagent. *N*-Terminally deprotected dipeptide **6** was obtained by hydrogenolytic removal of the Z-group from Z-Pro-Ser(*t*Bu)-O*t*Bu in 95% yield; this dipeptide was obtained in 80% yield by coupling of Z-Pro-OH and H-Ser(*t*Bu)-O*t*Bu in the presence of DIC and HOBt as condensation reagents. *C*-Terminally deprotected dipeptide **16** was obtained by hydrogenolytic cleavage of the benzyl ester from Boc-Leu-Pro-OBzl.
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