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Structural analysis of high affinity divalent phosphopeptide hybrids of spleen tyrosine kinase

M. Isabel Catalina[a], Frank J. Dekker[b], Rob M. J. Liskamp[b], Cees Versluis[a], Claudia S. Maier[a,1], Albert J. R. Heck[a,*]

[a] *Department of Biomolecular Mass Spectrometry, Bijvoet Centre for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands*
[b] *Department of Medicinal Chemistry, Bijvoet Centre for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, Utrecht, The Netherlands*

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This paper is dedicated to Helmut Schwartz on the occasion of his 60th birthday. We heartily congratulate him. A.J.R.H. would personally like to thank him for bringing with him his infectious enthusiasm for science, and mass spectrometry in particular.

Abstract

A set of synthetic phosphorylated peptidomimetic inhibitors of spleen tyrosine kinase (Syk), targeted towards its two tandem Src homology-2 (SH2) domains, was studied by nano-electrospray tandem mass spectrometry in both positive and negative ionisation mode. The design of the peptidomimetic compounds was based on the replacement of the intervening amino acid sequence of a Syk-binding di-phosphopeptide by non-peptide spacers based on either ethylene glycol or amino-propynyl-benzoate. Collision-induced dissociation (CID) spectra of the protonated molecular ions $[M + H]^+$ allowed full characterisation of the peptide hybrids. Preferred cleavage at the amide bond N-terminal to the adjacent polyethylene glycol (PEG) and the propynyl-benzoate (PrB) linkers was observed. In general, it thus appears that preferred sequential amino acid fragmentation takes place from the N-terminus up to the linker molecule followed by subsequent internal fragmentation starting at the C-terminus. Additionally, tandem CID spectra of the doubly de-protonated molecular ions $[M–2H]^{2-}$ of every compound showed the *m*/*z* 79/97 phosphate-specific ions *plus* a remarkably intense ion at *m*/*z* 297. The mechanism proposed for the *m*/*z* 297-ion occurrence goes through a five-membered ring formation giving an N-terminal pyroGlu structure as derived from MS^n spectra.

Keywords: Phosphorylation; Peptidomimetics; Syk inhibitors; SH2 domains; Fragmentation mechanisms

1. Introduction

Phosphorylation plays a crucial role in signal transduction within the cell. A family of functional modules often found in signalling proteins comprises the Src homology-2 (SH2) domains [1,2]. Molecular recognition of these domains is based on their binding to phosphotyrosine (pTyr) motifs of both catalytic proteins

* Corresponding author. Tel.: +31-30-2536797; fax: +31-30-2518219.
E-mail address: a.j.r.heck@chem.uu.nl (A.J.R. Heck).
[1] Present address: Department of Chemistry, Oregon State University, USA.

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(kinases, phosphatases, and lipases) and non-catalytic proteins (adapters and transcription factors) [3].

The intervention of signalling cascades by SH2 domain inhibitors is an interesting recent therapeutic avenue [4]. Since tyrosine-phosphorylated peptides are the natural substrates of SH2 domains, these peptides have been used as lead structures. Born out of the need to have peptide-like structures that are proteolysis-resistant and capable of crossing membrane boundaries, there is a strong interest in designing peptidomimetics mimicking their natural peptide lead. The cytoplasmic tyrosine kinase p72Syk (commonly referred as Syk, spleen tyrosine kinase) is a very interesting target because of its potential relevance to asthma and type-I allergic reactions [5]. An interesting feature of Syk is that it contains two SH2 domains in tandem [6]. Syk plays a crucial role in mast cell signalling via the high-affinity receptor for IgE (FcεRI). Syk binds to the phosphorylated immunoreceptor tyrosine-based activation motifs (ITAM) located on the cytosolic tail of the γ-subunit of FcεRI. The ITAM motif on the γ-chain of FcεRI for Syk has the amino acid sequence KADAV**pYTGL**NTRSQET**pYETL**KHEK. Phosphorylation of both tyrosine residues (**pY**) in this ITAM is required for high affinity binding to Syk [7].

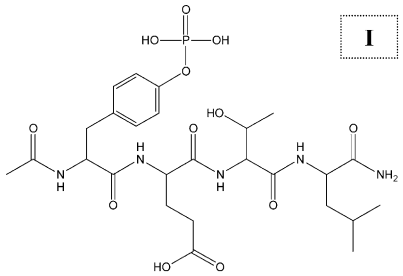
As mentioned above, a unique feature of Syk is that it contains two SH2 domains (in tandem). Binding of the tandem SH2 domain of Syk to the phosphorylated ITAM motif results in conformational changes which activates Syk's tyrosine kinase domain and initiates downstream signalling. The interaction of Syk with the ITAM motifs of the γ-chain of FcεRI is one of the critical steps during early activation of FcεRI signal transduction in mast cells. It is hypothesised that prevention of the interaction of Syk with FcεRI will interrupt mast cell activation and prevent allergic reactions. Thus, research efforts are directed towards the development of novel therapeutics that act as specific inhibitors of FcεRI Syk interaction. For this purpose, we have synthesised SH2 domain potential inhibitors belonging to three different classes. The first class represents selected amino acid residues of the consensus sequence of the γ-ITAM motif. The second group of constructs was peptoids or peptoid–peptide hybrids. Peptoid–peptide hybrids are oligomeric peptidomimetics that contain one or more N-substituted glycine residues (see Scheme 1, compound **II**). In these hybrids, the side chains of one or several amino acids are shifted from the α-carbon atom to the amide nitrogen. The third group comprised divalent constructs. Two short mono-phosphorylated peptides resembling the pY-binding patches (pYTGL and pYETL) of the phosphorylated ITAM motif are linked by non-peptide spacers. These non-peptide spacers are either ethylene glycol or amino-propynyl-benzoate based (see Scheme 1). The idea of this third group of compounds is to generate a divalent inhibitor, whereby the linker between the two pY-binding patches can be varied and thus optimised for Syk inhibition. Indeed, these novel constructs show affinities comparable to the interaction between the native Syk tandem SH2 domain and the di-phosphorylated ITAM [8,9].

In earlier reports of our laboratory, we described the use of soft ionisation techniques combined with collision-induced dissociation (CID) for the unambiguous characterisation of peptoids and isomeric peptide–peptoid hybrids [10–12]. In this paper, emphasis is directed towards a novel class of divalent SH2 potential inhibitors containing non-peptide spacers studied by both positive and negative nano-electrospray ionisation (nano-ESI) tandem mass spectrometry.

2. Experimental

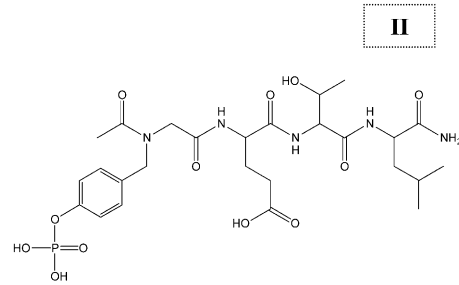
2.1. Peptide synthesis

Peptoids and di-phosphorylated peptidomimetic compounds were synthesised as previously described [8,9,13]. Briefly, compounds were prepared by solid-phase peptide synthesis (SPPS) on Rink amide resin using the Fmoc-based peptide chemistry. Couplings were accomplished with a mixture of the (Fmoc-) amino acid, benzotriazol-1-yloxy-tris(dimethylamino) phosphonium hexafluorophosphate (BOP) and *N*,*N*-diisopropylethylamine (DiPEA) in *N*-methyl-2-pyrro-



I

pYETL (645.2 amu)

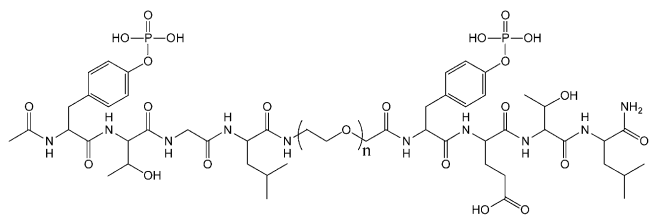


II

pY^{N}ETL (645.2 amu)

III

pYTGL **NTRSQET** pYETL (1975.8 amu)

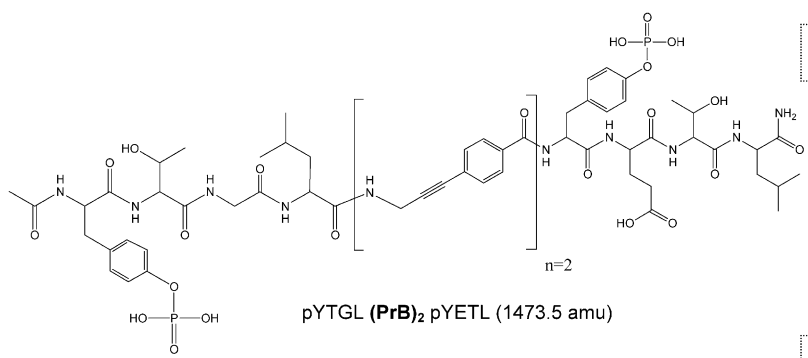


IV (n=6)

V (n=4)

pYTGL **(PEG)$_6$** pYETL (1480.6 amu)

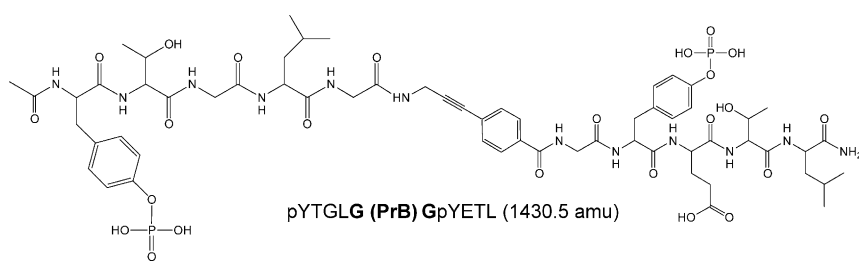
pYTGL **(PEG)$_4$** pYETL (1392.5 amu)



VI

pYTGL **(PrB)$_2$** pYETL (1473.5 amu)

VII



pYTGL**G (PrB) G**pYETL (1430.5 amu)

Scheme 1. Chemical structures of the compounds used in this study. pY^{N} represents a phosphotyrosine peptoid, PEG stands for polyehtylene glycol and PrB for amino-propynyl-benzoate.

lidinone (NMP) for 1 h. Deprotection of the Fmoc group was accomplished by treatment with 20% piperidine in NMP 2 × 8 min. After cleavage and deprotection, the peptides were purified by preparative reversed-phase high-performance chromatography (RP-HPLC) on a C18 column.

2.2. Mass spectrometers

Mass spectrometric measurements were performed on a quadruple time-of-flight (Q-TOF) instrument (Micromass, Manchester, UK) and/or on an ion trap (LC-Q) mass spectrometer (Finnigan, San Jose, CA). Both instruments were equipped with a nano-electrospray source: the Q-TOF instrument with a Z-nano-electrospray source (Micromass) and the LC-Q with a nano-flow electrospray source (Protana, Odense, Denmark); operating in either positive or negative ionisation mode. Nano-electrospray needles were made from borosilicate glass capillaries (Kwik-Fil, World Precision Instruments, Sarasota, FL) on a P-97 puller (Sutter instruments, Novato, CA). Needles were coated with a thin gold layer (∼500 Å) using an Edwards Scancoat Six sputter coater. All spectra were recorded from aqueous solutions of acetonitrile (50%) and formic acid (1%) at a peptide concentration of typically 5 μM.

In the case of the Q-TOF instrument, the potential between the nano-spray needle and the orifice of the mass spectrometer was normally set to 1.2 kV, and the cone voltage was varied from 30 to 80 V. In MS/MS mode, the quadruple was used to select the precursor ions, which were fragmented in the hexapole collision cell, generating product ions that were subsequently mass analysed by the orthogonal reflectron TOF mass analyser. For CID MS/MS measurements, the voltage over the hexapole collision cell was set to approximately 35 V, and argon was used as collision gas to a pressure of typically 11 kPa.

In the case of the LC-Q instrument, the potential between the nano-spray needle and the orifice of the mass spectrometer was normally set to 2.0 kV, and the capillary voltage/tube lens offset was varied from 20 to 80 V. CID spectra were recorded by first mass-selecting the whole isotope-envelope of the precursor ions in the ion trap, followed by resonant RF excitation collisional activation.

3. Results and discussion

3.1. Peptoids fragmentation pattern in positive ESI

Earlier reports from our laboratories showed mass spectrometry as a key player in the unambiguous characterisation of peptoids [10–12]. In the first two reports, fast atom bombardment (FAB) ionisation in combination with high-energy CID tandem mass spectrometry was used to obtain spectra of the protonated molecular ions of peptoids and their isomeric peptides. Increased abundances of the Y″ ions [14] in the spectra of peptoids were observed when compared with the corresponding isomeric peptides. In the third report [12], nano-electrospray ionisation in combination with low-energy CID was used to characterise a set of peptoid–peptide hybrids and peptoids derived from the phosphopeptide Ac-pYETL-NH_2 (Scheme 1, compound **I**). Isomeric compounds could be distinguished since characteristic fragment ions were observed in the peptoid spectra when compared to its isomeric peptide. For instances, a relatively intense *m*/*z* 187 ion would be apparent if a peptoid phosphotyrosine residue was incorporated (Scheme 1, compound **II**); this ion would come from the positively charged side chain of the peptoid phosphotyrosine residue [12].

3.2. Linker-peptidomimetics fragmentation pattern in positive ESI

3.2.1. PEG-linked divalent SH2 potential inhibitors

As reference compound, the naturally occurring high binding affinity peptide was chosen, i.e., the di-phosphorylated ITAM motif of sequence pYTGL**NTRSQET**pYETL (Scheme 1, compound **III**). The CID spectra of the singly protonated molecular ion of compound **III** (*m*/*z* 1977.8) showed the typical Y″- and B-type ion series [14] (data not

shown). For the PEG-linked divalent phosphopeptides, the intermediate amino acid sequence "NTRSQET" of compound **III** was replaced by an hexa- and a tetra-polyethylene glycol linker giving compounds **IV** and **V**, respectively (Scheme 1).

The singly protonated molecular ions of compound **IV** (pYTGL**(PEG)$_6$**pYETL, *m/z* 1481.6) and compound **V** (pYTGL**(PEG)$_4$**pYETL, *m/z* 1393.5) fragmented in a similar way when subjected to CID tandem MS. Roepstorff and Fohlman nomenclature [14] was used, and the complete linker region was considered as one residue. Both compounds gave the Y″-type ions ranging from Y''_8 to Y''_5, and the less intense B-type ions ranging from B_8 to B_6 (see Fig. 1a and b). In either of the MS/MS spectra, the Y''_5 ion was the far most intense fragment ion at *m/z* 925.4 and 837.3 for compounds **IV** and **V**, respectively. Notably, the Y''_5 ion is the one formed when all amino acids up to where the PEG linker starts are cleaved off, starting at the N-terminus. From this Y''_5 ion onwards, a B-type-like ion fragmentation pathway seemed to occur (Scheme 2a). In the spectra we did observe these internal fragment ions generated from the Y''_5 ion, being the $(B_8Y_5)_4$, $(B_7Y_5)_3$ and $(B_6Y_5)_2$ ions at *m/z* 795.4, 694.3 and 565.2 for compound **IV**, and at *m/z* 707.2, 606.2 and 477.2 for compound **V**, where $(B_rY_s)_n$ is an internal fragment ion, and the suffixes *r* and *s* indicate the bonds cleaved, counting from the N- and C-termini, respectively. The subscript *n* indicates the number of amino acid residues retained in the internal fragment ion. As for normal (underivatised) peptides, no $(B_5Y_5)_1$-type ion was found [14,15].

Another interesting ion at *m/z* 600.3 was observed in the MS/MS spectra of both compounds **IV** and **V**, this ion can be considered as a D-type ion with the structure depicted in Scheme 2b. For conventional peptides, D-type ions are usually not very abundant and typically formed via high-energy processes. The relative high abundance of the D_5 ion is, therefore, most likely a consequence of the particular chemical structure as it results from the cleavage between the peptide and the linker moiety. This D_5 ion seems to have a highly basic site at its C-terminus promoting the formation of internal fragments giving a series of Y″-type ions. These internal fragments are $(D_5Y_8)''_3$, $(D_5Y_7)''_2$ and $(D_5Y_6)''_1$ at *m/z* 315.3, 214.3 and 157.3, respectively, where $(D_rY_s)''_n$ is an internal fragment ion, and the suffixes *r* and *s* indicate the bonds cleaved, counting from the N- and C-termini, respectively. The subscript *n* indicates the number of amino acid residues retained in the internal fragment ion.

The PEG linkers were found back as protonated amino-polyethylene glycol ions at *m/z* 282.3 and *m/z* 194.3, for compounds **IV** and **V**, respectively.

3.2.2. PrB-linked divalent SH2 potential inhibitors

For the PrB-linked divalent phosphopeptides the intermediate amino acid sequence "NTRSQET" of compound **III** was replaced by a di-amino-propynyl-benzoate $(PrB)_2$ and a Gly-amino-propynyl-benzoate-Gly (G(PrB)G) linker giving the compounds **VI** and **VII** (Scheme 1), respectively. As for the former PEG-linked peptides, the $(PrB)_2$ and (PrB) linker regions were, respectively, considered as one residue.

The singly protonated molecular ion of compound **VI** (pYTGL**(PrB)$_2$**pYETL, *m/z* 1474.5) fragmented giving a series of B-type ions, followed by loss of H_2O, namely B_8, B_7, B_6, B_4 and B_3, as well as the Y″-type ions ranging from 8 to 5 (see Fig. 2a). Interesting is that all Y″ ions could form internal B-type fragment ions, resulting in the $(B_rY_s)_n$ internal fragment ions (for nomenclature see above) as shown in the spectrum of Fig. 2a. From the Y''_8 ion, the $(B_8Y_8)_7$, $(B_7Y_8)_6$ and $(B_6Y_8)_5$ internal fragments at *m/z* 1059.5, 958.4 and 829.3, respectively, were obtained; as from the Y''_7 ion, the $(B_8Y_7)_6$, $(B_7Y_7)_5$ and $(B_6Y_7)_4$ internal fragments at *m/z* 958.4, 857.3 and 728.3, respectively; as from the Y''_6 ion, the $(B_8Y_6)_5$ ion at *m/z* 901.3; and as from the Y''_5 ion, the $(B_8Y_5)_4$, $(B_7Y_5)_3$ and $(B_6Y_5)_2$ ions at *m/z* 788.3, 687.2 and 558.2, respectively, were also observed. In order to make clearer the internal fragments which are formed from the Y″ ions we have depicted in Scheme 2c the structure of the Y''_5 ion and its derived B-type internal fragments as an example. The Y''_5 ion is one of the most intense Y″ ions (Fig. 2a) and is formed by the removal of all the N-terminal amino acid residues up to the linker region.

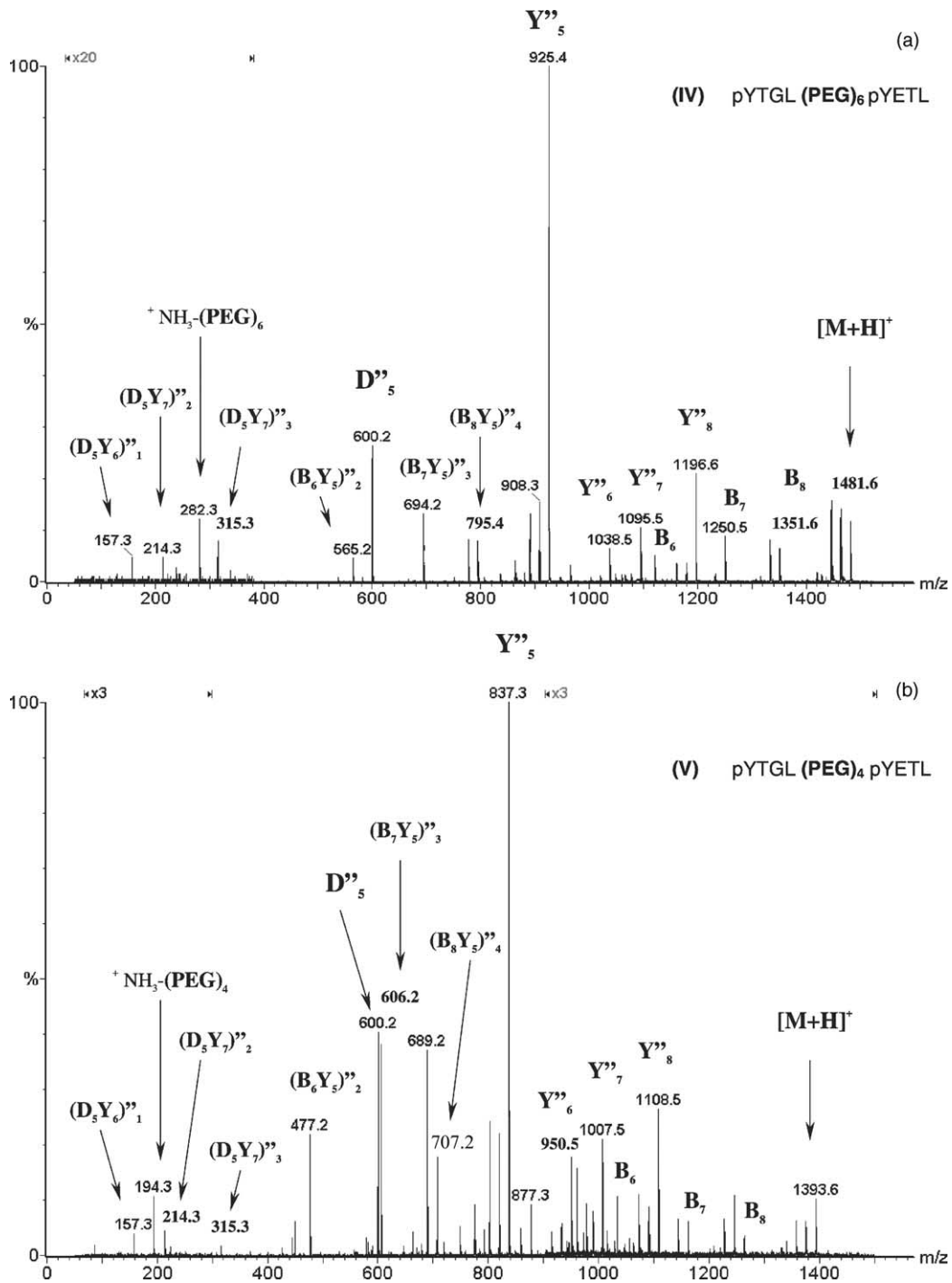
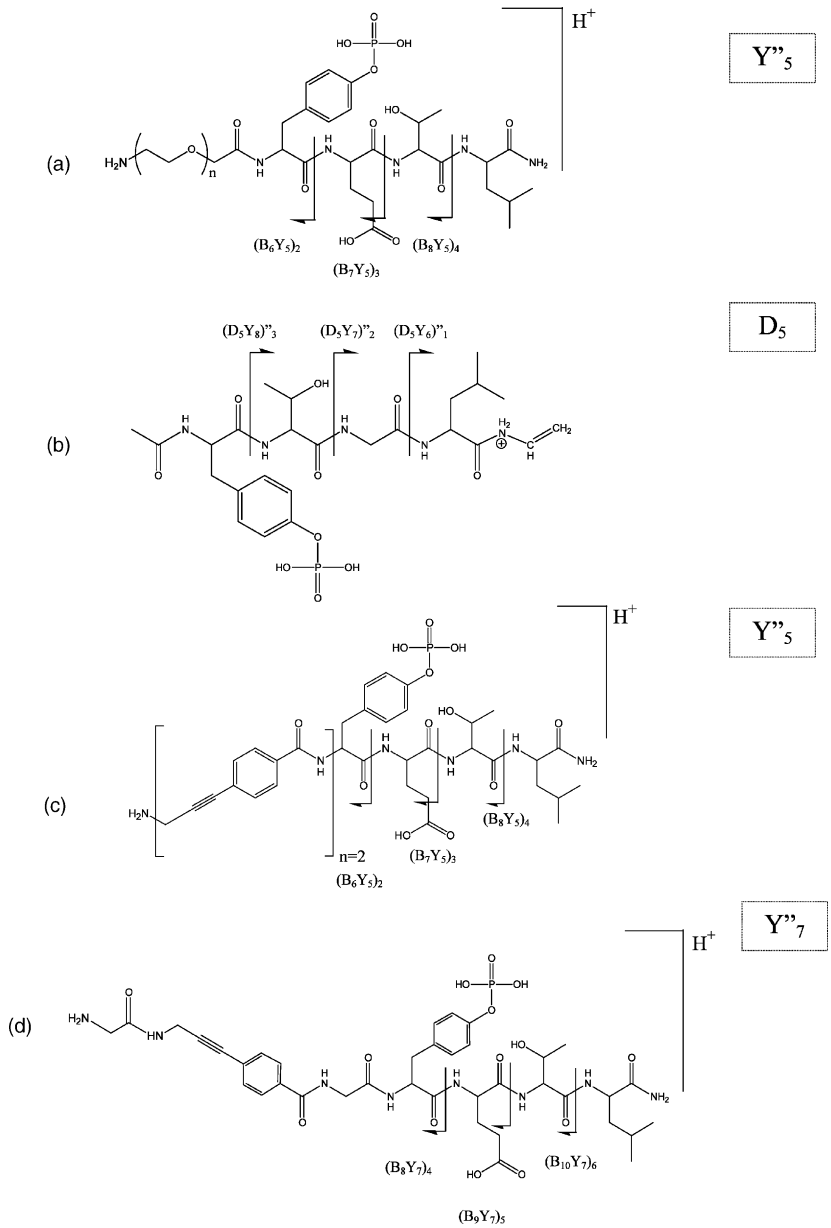


Fig. 1. Positive-ESI MS/MS spectra of the singly protonated PEG-linked divalent SH2 potential inhibitors. (a) Spectra of compound **IV** (hexa-PEG linked), and (b) spectra of compound **V** (tetra-PEG linked).



Scheme 2. Abundant fragment ions formed by selective cleavage of all amino acids up to the linker moiety. (a) Y''_5 is the ion formed when all amino acids, up to where the PEG linker starts, are cleaved off at the N-terminus. This ion fragments further from the C-terminus leading to the $(B_rY_s)_n$ ions. (b) D_5 is the ion formed when all the N-terminal amino acids including the PEG linker are cleaved off. This ion fragments further from the N-terminus leading to the depicted $(D_rY_s)''_n$ ions. (c) Y''_5 is the ion formed when all amino acids, up to where the $(PrB)_2$ linker starts, are cleaved off at the N-terminus. This ion fragments further from the C-terminus leading to depicted $(B_rY_s)_n$ ions. (d) Y''_7 is the ion formed when all amino acids, up to where the G-(PrB) linker starts, are cleaved off at the N-terminus. This ion fragments further from the C-terminus leading to the depicted $(B_rY_s)_n$ ions.

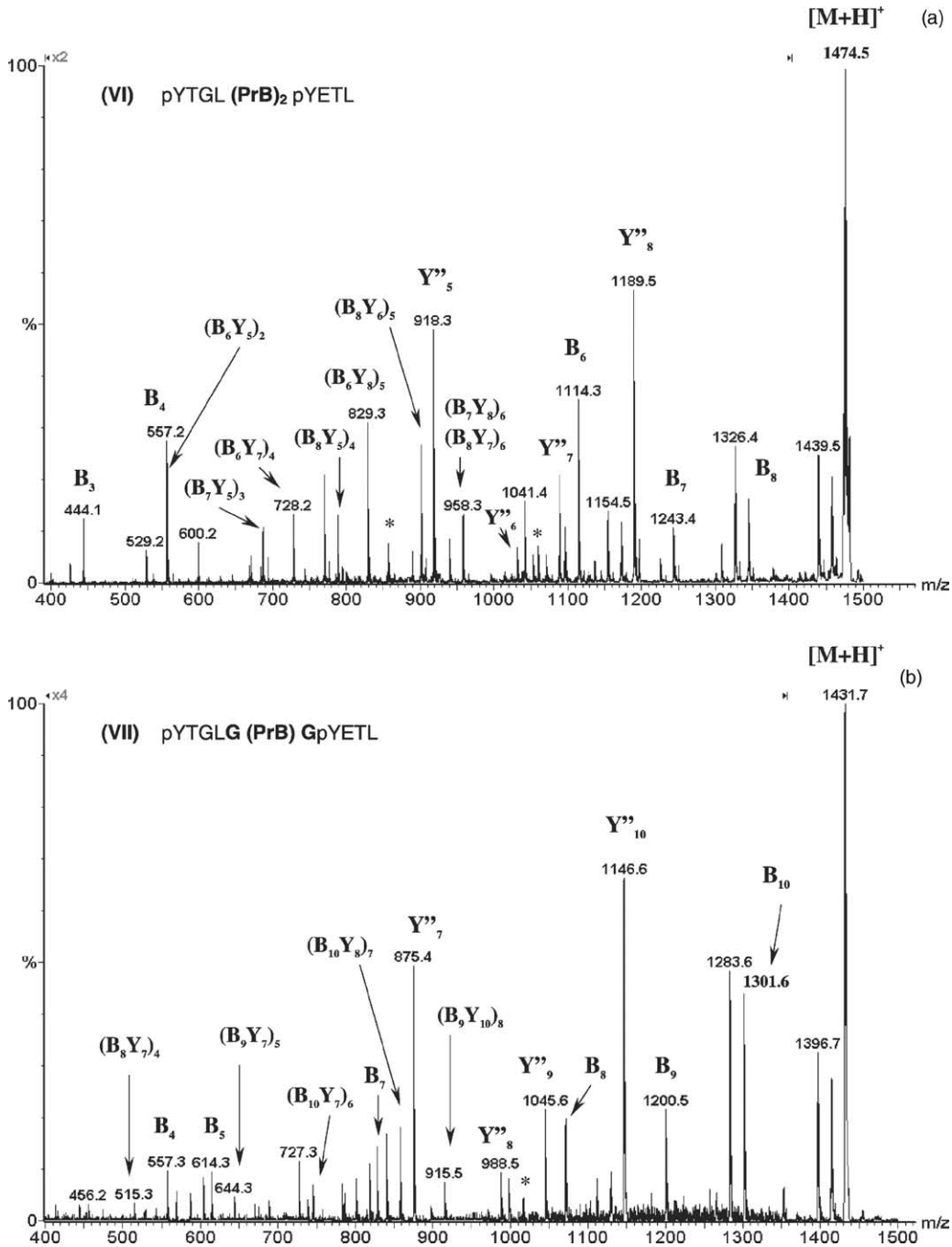


Fig. 2. Positive-ESI MS/MS spectra of the singly protonated PrB-linked divalent SH2 potential inhibitors. (a) Spectra of compound **VI** (di-amino-propynyl-benzoate linked). The asterisks locate the $(B_8Y_8)_7$ ion at *m*/*z* 1059.5 and the $(B_7Y_7)_5$ ion at *m*/*z* 857.3. (b) Spectra of compound **VII** (Gly-amino-propynyl-benzoate-Gly linked). The asterisk locates the $(B_{10}Y_{10})_9$ ion at *m*/*z* 1016.4.

The singly protonated molecular ion of compound **VII** (pYTGL**G(PrB)G**pYETL, *m/z* 1431.5) fragmented giving a series of B ions, followed by loss of H_2O, namely B_{10}, B_9, B_8, B_7, B_5, B_4 and B_3, as well as the Y″-type ions ranging from 10 to 7 (see Fig. 2b). As for the former PrB-linked compound, all these Y″ ions formed internal B-type fragment ions as shown in Fig. 2b. Namely, from ion Y''_{10}, the $(B_{10}Y_{10})_9$, $(B_9Y_{10})_8$ and $(B_8Y_{10})_7$ internal fragments at *m/z* 1016.4, 915.4 and 786.4, respectively; from the Y''_9 ion, the $(B_{10}Y_9)_8$ ion at *m/z* 915.5; from the Y''_8 ion, the $(B_{10}Y_8)_7$ ion at *m/z* 858.4, and, from the Y''_7 ion, the $(B_{10}Y_7)_6$, $(B_9Y_7)_5$ and $(B_8Y_7)_4$ internal fragments at *m/z* 745.4, 644.4 and 515.3, respectively. For nomenclature see above.

In order to clarify the internal fragments which are formed from the Y″ ions we have depicted in Scheme 2d the structure of the Y''_7 ion and its derived B-type internal fragments. The Y''_7 ion is one of the most intense Y″ ions (Fig. 2b) and that it is formed by the removal of all the N-terminal amino acid residues up to the linker region.

3.3. Linker-peptidomimetics fragmentation pattern in negative ESI

As a typical example, the CID tandem mass spectrum of the $[M–H]^-$ ion (*m/z* 644.3) of peptide **I** (pYETL) is shown in Fig. 3a. The major peaks are at *m/z* 79 and 97, corresponding to the ions $[PO_3]^-$ and $[H_2PO_4]^-$, respectively. A third major peak corresponds to the *m/z* 297 ion. The CID tandem mass spectra of the $[M–2H]^{2-}$ ion of the di-phosphorylated compounds **V** and **VI** are depicted in Fig. 3b and c. As for the reference peptide **I**, the most abundant ions correspond to *m/z* 79 and 97 from phosphate-specific marker ions. Intriguingly, a third major peak corresponding to the *m/z* 297 ion also appears in all the different spectra. As this fragmentation pattern was observed for the $[M–2H]^{2-}$ ions of all compounds we proceeded to investigate the structure of the *m/z* 297 ion in MS^n experiments with the prospect of finding a potential new marker ion. All peptide/peptidomimetics share the consensus amino acid sequence pYETL, which seemed, at first glance, the candidate sequence from which the ion *m/z* 297 would originate.

The MS^2 spectrum of the de-protonated peptide **I** showed the prominent ions with *m/z* 546, 341 and 297 (Fig. 4a). Subsequent MS^3 on *m/z* 341 resulted in a singly predominant ion at *m/z* 297 as shown in Fig. 4b. Further MS^4 on *m/z* 297 resulted in an 18-(H_2O) loss, giving the *m/z* 279 ion (spectrum not shown). MS^5 on the latter ion disclosed the *m/z* 151 and 127 fragment ions (Fig. 4c).

The *m/z* 546 ion obtained from the MS^2 fragmentation came from the 98 mass loss (presumably from

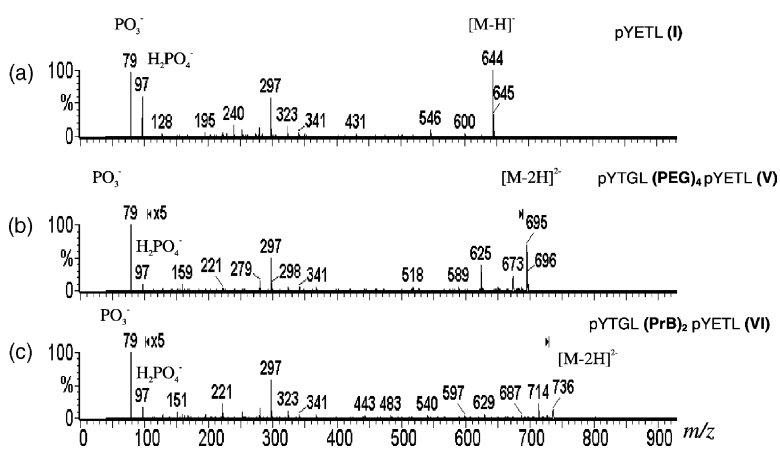


Fig. 3. Negative-ESI MS/MS spectra. The CID spectrum of the $[M–H]^-$ ion of reference compound **I** is shown in (a). CID tandem mass spectra of the $[M–2H]^{2-}$ ions of the di-phosphorylated compounds **V** and **VI** is shown in (b) and (c), respectively.

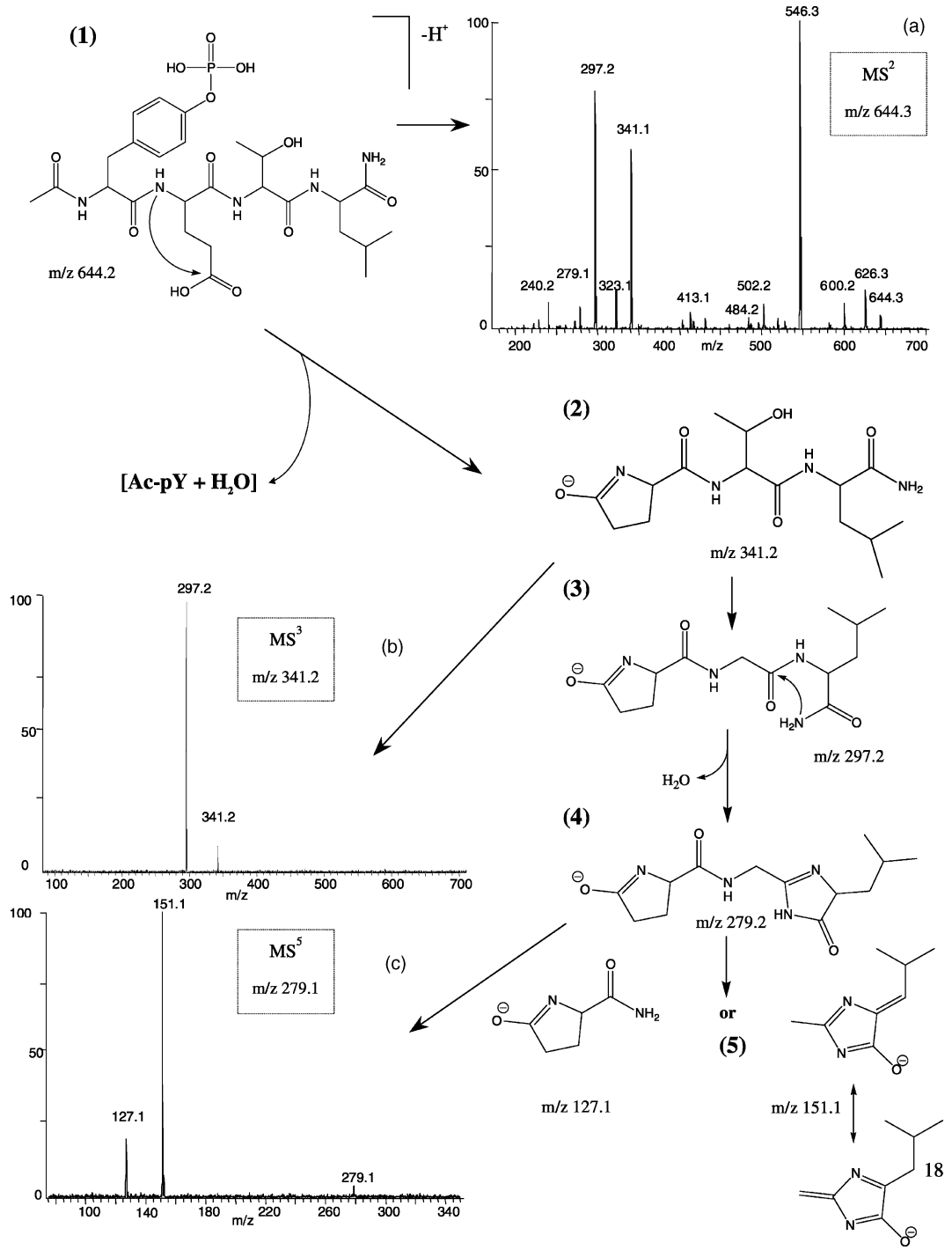


Fig. 4. MS^n experiments on the pYETL $[M–H]^-$ ion. Selected CID spectra and proposed fragmentation mechanism (see text for details).

HPO_3 and H_2O losses [16]) from the de-protonated peptide ion (*m*/*z* 644); further, MS^3 on this ion (*m*/*z* 546) showed *m*/*z* 323 and 240 as predominant ions. Following MS^4 on *m*/*z* 323 gave the *m*/*z* 279 ion, which ultimately resulted in the *m*/*z* 151 and 127 ions by MS^5. The conclusion to be drawn from the MS^n spectra of the *m*/*z* 546 ion was that the *m*/*z* 297 fragment under investigation lacked the phosphate. The negative charge was to be held in other acidic group/s.

The proposed mechanism for the formation of the *m*/*z* 297 ion is the one represented in Fig. 4. The fragmentation proceeds with a five-centred nucleophilic attack of the amide nitrogen of Glu at the carboxyl carbon (**1**) forming a pyroGlu structure, which fragments into an N-terminal pyroGlu at *m*/*z* 341 (**2**) and releases Ac-pY/H_2O as neutrals, as described previously [17]. Loss of mass 44 follows, corresponding to the characteristic loss of the Thr side chain [18,19], giving the *m*/*z* 297 ion (**3**). A second five-centred nucleophilic attack of the amide nitrogen at the C-terminus to the contiguous carbonyl group will result in the 18-(H_2O) loss giving the *m*/*z* 279 ion (**4**). Subsequent fragmentation will result in two cyclic ions of *m*/*z* 127 (pyrrole ring) and 151 (imidazole ring), respectively, as depicted in Fig. 4 (**5**).

4. Conclusions

A set of synthetic phosphorylated peptidomimetic inhibitors of Syk tandem SH2 domain was studied by nano-electrospray tandem mass spectrometry in both positive and negative ionisation mode in order to elucidate their fragmentation pattern with view to large-scale peptide libraries synthesis and identification. CID spectra of the protonated molecular ions $[M+H]^+$ allowed full characterisation of the peptide hybrids. Multiple cases of charge sequestering with enhanced amide bond-cleavage were observed. Multiple internal fragment ions, namely (B_rY_s) and $(D_5Y_s)''$ ions, were also detected. Most of the former internal ions derived from the very abundant Y'' ion generated upon loss of the N-terminal amino acid residues up to the linker region, resulting in a very characteristic fragmentation pattern of these hybrid compounds.

Tandem CID spectra of the doubly de-protonated molecular ions $[M–2H]^{2-}$ showed a common pattern for all the compounds, i.e., the phosphate-specific *m*/*z* 79/97 ions *plus* a remarkably intense ion at *m*/*z* 297. The mechanism proposed for the *m*/*z* 297-ion occurrence proceeds with a five-centred nucleophilic attack of the amide nitrogen of Glu at the carboxyl carbon forming a pyroGlu structure as determining step. The phosphate group-specific ions in the negative ion mode constitute an excellent marker for phosphopeptides, as well as the *m*/*z* 297 ion represents an excellent marker for these compounds in the negative ion mode.

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