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Petasis-Ugi ligands: New affinity tools for the enrichment of phosphorylated peptides

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ABSTRACT

Affinity chromatography is a widespread technique for the enrichment and isolation of biologics, which relies on the selective and reversible interaction between affinity ligands and target molecules. Small synthetic affinity ligands are valuable alternatives due to their robustness, low cost and fast ligand development. This work reports, for the first time, the use of a sequential Petasis-Ugi multicomponent reaction to generate rationally designed solid-phase combinatorial libraries of small synthetic ligands, which can be screened for the selection of new affinity adsorbents towards biological targets. As a proof of concept, the Petasis-Ugi reaction was here employed in the discovery of affinity ligands suitable for phosphopeptide enrichment. A combinatorial library of 84 ligands was designed, synthesized on a chromatographic solid support and screened *in situ* for the specific binding of phosphopeptides binding human BRCA1C-terminal domains. The success of the reaction on the chromatographic matrix was confirmed by both inductively coupled plasma atomic emission spectroscopy and fluorescence microscopy. Three lead ligands were identified due to their superior performance in terms of binding capacity and selectivity towards the phosphorylated moiety on peptides, which showed the feasibility of the Petasis-Ugi reaction for affinity ligand development.

1. Introduction

There is great interest in the development of affinity ligands, which assist in the specific capture, purification, identification and quantification of numerous biopharmaceuticals, disease-related proteins and biomarkers [1,2]. Affinity ligands are classified in two major classes: biospecific and pseudobiospecific [3]. Biospecific ligands are the natural binding partners of a particular target. Pseudobiospecific ligands are chemically defined molecules, either biological (e.g. peptides, aptamers) or synthetic (e.g. hydrophobic, thiophilic, mixed-mode, meta-chelating ligands). Biomimetic ligands are engineered molecules with improved features which belong to one of the subclasses of pseudobiospecific ligands (e.g. engineered protein domains, de novo designed ligands) [3,4]. This class of ligands has been developed to overcome drawbacks of their naturally-occurring templates, presenting high chemical resistance to cleaning-in-place and sterilization-in-place procedures, high scalability, high binding capacities and low production costs [3,5].

Multicomponent reactions, which include the Ugi reaction and the Petasis borono-Mannich reaction, are one-pot reactions that consist of the combination of three or more compounds to yield a single product. The resulting product retains all of the atoms of the starting materials, excepting the condensation products [6]. The Ugi reaction was reported in 1959 by Ivar Ugi and co-workers, and consists of the condensation between a primary or secondary amine, an aldehyde or ketone, a carboxylic acid and an isonitrile [7]. The Ugi reaction has been used for the development of affinity ligands for the purification of a myriad of target molecules, such as antibodies [8], glycoproteins [9], green fluorescence protein [10], among others. The Petasis borono-Mannich reaction consists of the addition of an aldehyde, an amine, and a boronic acid, named after its discovery by Nicos A. Petasis in 1993 [11]. The reaction has been used for the synthesis of several compounds, namely α-amino acids, amino alcohols, 2-hydroxymorpholines and 2H-chromenes. Secondary amines are the most reactive, followed by bulky primary amines, but the employment of tertiary amines has been reported as well [12–14]. The presence of a hydroxyl group in the amine or aldehyde components is vital for the activation of the boronic acid. Therefore, aldehydes comprising hydroxyl or carboxyl

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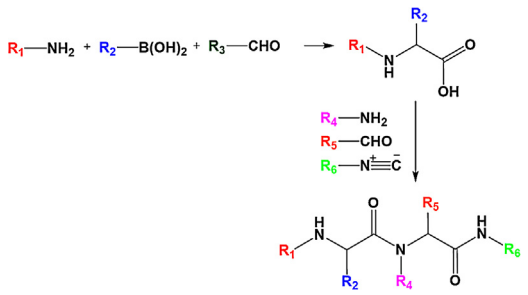

Fig. 1. Sequential Petasis-Ugi multicomponent reaction. Petasis reaction involves an amine, a boronic acid and an aldehyde. The carboxylic acid generated in the Petasis reaction can then be used as one of the components of the Ugi reaction – a four-component one-pot reaction between a carboxylic acid, an amine, an aldehyde and an isocyanide.

groups, such as glycolaldehyde, glyoxylic acid and salicylaldehyde are commonly reported [13,15,16]. Petasis and Zavialov have also successfully used α-keto acids, such as pyruvic acid, instead of the aldehyde component [14].

As illustrated in Fig. 1, the Petasis product generates a free carboxylic acid (deriving from the aldehyde component), which can be further employed as a component in other reactions, such as the Ugi reaction. The sequential Petasis-Ugi reaction increases molecular diversity, through the incorporation of a higher number of functional groups when compared to the individual Ugi and Petasis reactions. Although the Petasis-Ugi reaction is known since 2003 [17], it has never been performed directly on chromatographic supports and explored as a platform for the discovery of ligands for purification and enrichment purposes. The aim of this work is to develop novel synthetic affinity ligands for the capture of relevant biological targets using a high-throughput platform based on the sequential Petasis-Ugi multicomponent reaction (Fig. 1). The resultant adsorbents are target-oriented, inexpensive, and highly stable in comparison to biological affinity ligands [3]. As a proof-of-concept, phosphopeptides were selected as model targets due to the need to isolate and concentrate disease-related phosphopeptides in biological samples.

2. Materials and methods

2.1. Materials

All reagents were of the highest purity available and the solvents were pro-analysis. 1-Pyrenemethylamine hydrochloride, 2-(ethylthio)ethylamine, 2-ethylhexanal, 3-(methylthio)propionaldehyde, 3-thienylboronic acid, 4-imidazolecarboxaldehyde, agmatine sulfate salt, amino-2-propanol, ammonium bicarbonate, ammonium hydroxide solution (NH_4OH), cadaverine dihydrochloride, epichlorohydrin, glyoxylic acid monohydrate, histamine, indole-3-carboxaldehyde, isopentylamine, isopropyl isocyanide, m-xylylenediamine, *N,N*-dimethyl-4,4′-azodianiline, ninhydrin, phenethylamine, phenol, phenylacetaldehyde, phenylboronic acid, potassium cyanide, propionaldehyde, pyrene-1-boronic acid, pyridine, sodium bicarbonate ($NaHCO_3$), sodium thiosulfate, tetrahydrofuran-3-carboxaldehyde solution, tris(hydroxymethyl)aminomethane, tryptamine and tyramine were acquired from Sigma-Aldrich (Sintra, Portugal). Methanol (MeOH) was obtained from Carl Roth (distributed by BetaLab Lda., Queluz, Portugal), 4-aminomethylphenylboronic acid hydrochloride from Synthonix (Wake Forest, NC, USA), and acetic acid glacial from Pronalab (Lisbon, Portugal). Dimethylformamide (DMF) was purchased from VWR (Carnaxide, Portugal). Acetic anhydride, ethanol absolute PA, hydrochloric acid 37% (HCl), nitric acid, sodium acetate and sodium hydroxide (NaOH) were acquired from Panreac (distributed by Fisher Scientific, Loures, Portugal). The peptides Ser-Gln-Val-Phe-Pro-Trp (SW6), pSer-Gln-Val-Phe-Pro-Trp (SW6-P), Thr-GlnVal-Asp-Ala-Trp (TW6), pThr-Gln-Val-Asp-Ala-Trp (TW6-P), Tyr-Glu-Glu-Ile-Pro-Trp (YW6), and pTyr-GluGlu-Ile-Pro-Trp (YW6-P) were > 98% pure and were purchased from Genecust (Dudelange, Luxembourg) and Caslo (Lyngby, Denmark). Sepharose™ CL–6B was acquired from GE Healthcare (Carnaxide, Portugal).

2.2. Instrumentation

Inductively Coupled Plasma – Atomic Emission Spectroscopy (ICP-AES) analysis for the detection of boron and sulphur in ligand-functionalized agarose was conducted in a Horiba Jobin-Yvon Ultima Spectrophotometer at an impressed potential of 1200 kW (Horiba ABX SAS, Portugal). Fluorescence microscopy assays used an Olympus BX51 microscope (400× magnification), a U-MWB filter (λ_{ex} = 460–490 nm; λ_{em} = 515–700 nm), an UPlanFL objective, an U-RFL-T lamp, and Cell View software for monitoring (Olympus, Portugal). Fluorescence and absorbance readings in 96-well microplates were conducted in a Tecan F200 Microplate Reader using a λ_{ex} = 280 (20) nm–λ_{em} = 340 (35) nm filter, and 560 (10) nm filter, and a 280 (5) nm filter (Tecan, Grupo Taper, Portugal). The values within brackets refer to the bandwidth of the filters.

2.3. Methods

2.3.1. Epoxy-activation of agarose

Sepharose™ CL–6B was washed with distilled deionised water (dd water) (10 × resin volume) in a sinter funnel using vacuum suction, and then resuspended in dd water (1 mL/g moist agarose) and 10 M NaOH (0.04 mL/g moist agarose). The suspension was incubated for 30 min at 34 °C with orbital shaking (230 rpm). Epichlorohydrin was then added in the proportion of 0.072 mL/g moist agarose and the mixture was left for another 3 h at 34 °C in the orbital shaker (230 rpm). In the end, the resin was washed with dd water (10 × resin volume). In order to determine the amount of epoxy groups, 1 g of epoxy-activated agarose was incubated with 3 mL of an aqueous solution of 1.3 M sodium thiosulfate for 20 min at room temperature (RT) with agitation. Epoxy groups were then quantified by titration with 0.1 M HCl. Typical values were 21 ± 1 μmol epoxy/g moist agarose.

2.3.2. Functionalization of agarose with 4-aminomethylphenylboronic acid

4-Aminomethylphenylboronic acid hydrochloride (3 equivalents molar excess relative to epoxy groups; 3 × 21 μmol/g moist agarose) was dissolved in dd water with an equivalent of NaOH, in order to make the amine reactive towards the epoxy groups on agarose. Then, the boronic acid solution was added to the epoxy-activated agarose (1 mL solution/g moist agarose) and the reaction was left for 24 h at 60 °C with orbital agitation (200 rpm). The resin was thoroughly washed with dd water by vacuum filtration.

2.3.3. Petasis reaction on boronic acid modified agarose

Agarose functionalized with 4-aminomethylphenylboronic acid was washed with 25% (v/v) ethanol/dd water (5 × resin volume) and 50% (v/v) ethanol/dd water (5 × resin volume). A mixture of 1-pyrenemethylamine hydrochloride and glyoxylic acid monohydrate (5 molar equivalents of each compound in excess relative to the epoxy) in 50% (v/v) ethanol/dd water was added and the reaction mixture was incubated for 48 h at 60 °C with orbital shaking (1 mL/g moist resin). NaOH was added to the amine component in the proportion of 1 molar equivalent NaOH/ molar equivalent of 1-pyrenemethylamine hydrochloride. 1-Pyrenemethylamine