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Catch-enrich-release approach for amine-containing natural products†

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Amine-containing natural products are an important class of therapeutic compounds. Herein, we report a chemoselective approach to catch and enrich amine-containing natural products, and release them as underivatized compounds. The strategy exploits the selectivity of the enzyme legumain for the specific release of amine-containing natural products.

For centuries, natural products (NPs) have been exploited for their therapeutic properties and have had a crucial impact on human health.[1,2] Their success is related to their structural diversity and the large chemical space that they occupy compared to synthetic compounds.[3–6] The discovery of novel bioactive NPs is and will continue to be essential for the treatment of current diseases[7–9]

A phenotypic screening followed by a bioactivity-guided fractionation represents the classical approach to identifying active NPs. The process includes (1) the selection of an organism, (2) the extraction of the compounds from the biomass, (3) the separation of the active compound by the selection of active fraction(s), and (4) the structure elucidation of the isolated compound.[10] During the process, several challenges might be encountered, such as the accessibility of the biomass, the isolation/exclusion of known NPs, the low production of the active compound, and the cost of the isolation and structure elucidation procedure.[3,11,12]

Novel strategies have been developed recently to facilitate the identification of known NPs (dereplication). One consists of genome mining (*e.g.* antiSMASH) to predict the presence of NPs in a crude extract.[13–16] Other approaches are based on metabolomics and use tandem mass spectrometry analysis or high-resolution mass spectrometry (HRMS) to identify NPs using platforms such as the Global Natural Products Social Molecular Networking (GNPS).[17–20] Those powerful approaches have the advantage of building upon growing databases while requiring only small amounts of analytical samples.

Another emerging technology consists of chemoselective approaches for the enrichment of NPs possessing a specific functional group.[21–25] This concept is based on a selective catching *via* a derivatisation agent[25,26] attached to a solid support and the release of the (un)derivatised NPs triggered by an external stimulus.[27] Currently, several methods have been developed for the catch and release of NPs possessing amine,[28–32] hydroxy,[33–36] carbonyl,[28,29,37–40] carboxylic acid,[28,29,34] thiol,[28,29,41] and alkyne[42,43] functional groups. Only a few strategies afforded an underivatized NP as the released compound by using siloxyl-functionalized resin[33–36] or by utilizing a pyridyl disulfide activation group.[41]

Herein, we are reporting a chemoselective catch-enrich-release approach to obtain underivatized amine-containing NPs. Our methodology exploits the advantage of streptavidin-coated magnetic beads and the high specificity of the enzyme legumain.[44] The amine functional group is highly abundant in natural products[45] including many bioactive molecules.[46] The importance of this functional group is highlighted by the discovery of the neurotransmitter serotonin (**1**, Fig. 1),[47] the stimulant pseudoephedrine (**2**),[48] and the neurotoxin anatoxin-a (**3**).[49]

Unlike other chemoselective approaches for amine-containing compounds, we envisioned that the chemoselectivity of our enrichment strategy would arise from the releasing step instead of focusing on the initial catch.[28,29,31,40] To obtain an underivatized compound, we sought to use an enzymatic cleavage as the last step of our protocol. The asparaginyl endopeptidase

Fig. 1 Examples of amine-containing natural products.

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Fig. 2 (A) Design of the probe for the catch, enrich and release of amine-containing NPs. (B) Structure of the probe **4** with asparagine in position P1.

legumain was selected since it is commercially available, has high tolerance at amino acid P1′[50] and has a selective recognition for the residues at P1–P4.[51]

The design of the desired probe includes a biotin anchor for the enrichment process, an amino acid sequence for the selective enzymatic release, and a carboxylic acid group for the catch of the amine-containing compounds (Fig. 2A). The first approach was based on (D)-biotin and used L-Ala-L-Ala-L-Ala-L-Asn recognition sequence for legumain. The probe **4** was synthesised by solid-phase peptide synthesis (SPPS) and its reactivity towards amine-containing compounds was investigated. The carboxylic acid was activated using *N*-hydroxysuccinimide and, unfortunately, the product was not stable and several by-products were observed. We hypothesised that the amide of Asn side chain might react with the activated electrophile. Similar results were observed in a previous study.[52]

To circumvent the undesired reactivity of the Asn side chain, we turned our attention to an approach using Asp instead. The design of the second approach was inspired by a recent study from Drag and co-workers, where the legumain recognition sequence was optimized for Asp.[53] The new probe **5** consisted of biotin followed by the linker 6-aminocaproic acid (6-Ahx) and the amino sequence D-Tyr-L-Tic-L-Ser-L-Asp(OAll). The probe **5** was synthesized by SPPS and was converted to the switchable fluorescent probe **6** using HATU and 7-amino-4-methylcoumarin (AMC, 7) followed by removing the allyl group using the Tsuji–Trost condition (Fig. 3A).[54] The efficacy of legumain-mediated cleavage was investigated by treatment of probe **6** with 15 ng mL^{-1} of the enzyme legumain. We were pleased to observe a release of 34% of the fluorophore after 130 minutes (Fig. 3B and Fig. S1, ESI†). Furthermore, the efficacy of legumain was also evaluated with the commercially available substrate Z-L-Ala-L-Ala-L-Asn-AMC, which yielded a recovery 84% (Fig. S1, ESI†). The amino acid at position P1 is hypothesized to account for the difference in yield between both compounds.

With this encouraging result, the enrichment strategy was tested by incubating probe **6** with streptavidin-coated magnetic beads followed by enzymatic cleavage. The release of AMC (7) was not observed, and we concluded that the proximity of the streptavidin hindered legumain from accessing the recognition

Fig. 3 (A) Structure of probe **5** and fluorescent probe **6**. (B) Kinetic release of AMC (**7**) while incubating probe **6** with legumain at a concentration of 15 ng mL^{-1}.

sequence. Therefore, it was necessary to release the probe from the solid support before performing the enzymatic cleavage.

It was crucial to establish a protocol using mild conditions for the release of the probe from the solid support to preserve the structural integrity of natural products during the procedure. Therefore, a recent method from Bearden and co-workers was adapted with the use of a mixture of phenol and chloroform for the release of biotinylated compounds from streptavidin-coated beads (Fig. S2, ESI†).[55] The conditions were applied to our system and an efficient release of probe **6** was observed. However, the release of AMC (7) was not observed after attempting enzymatic cleavage. We hypothesized that the remaining traces of phenol might alter the structure of legumain,[55] therefore, we stopped investigating this approach.

To encourage the competitive elution of biotinylated compounds from the streptavidin beads, probe analogues containing biotin derivatives, which have a weaker affinity towards streptavidin as compared with biotin, can be applied. Using this approach, the compounds can be eluted with a biotin solution. Biotin sulfone (**8**), desthiobiotin (**9**), and *N*′3-ethyl biotin (**10**) were selected as biotin derivatives and the probes bearing these anchors were synthesized by SPPS (Fig. 4A and Fig. S3A, ESI†). Both derivatives **8** and **9** were synthesized following the reported procedures[26g,56] while the tyrosine and the serine were protected to avoid side reactions. The attachment and the release from the solid support were performed with the three probes, and their concentrations after their release from the beads with a 5 mM biotin solution were analysed by UHPLC-MS. The biotin sulfone probe **11** showed the most promising result with a 27% recovery after a 10-minute incubation. The calculated recovery yield for the desthiobiotin analogues **12** and the *N*′3-ethyl biotin probe **13** were 12% and 8%, respectively(Fig. 4 and Fig. S3B, ESI†).

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Fig. 4 (A) Structure of the biotin sulfone-based probe **11**, the desthiobiotin-based probe **12**, and the *N*′3-ethyl biotin-based probe **13**. Abbreviation: All for allyl and PPG for propargyl functional group. (B) Schematic representation of the complete protocol for the catch-enrich-release of amine-containing NPs.

Then, we investigated the deprotection of the propargyl and the two allyl groups in the recognition sequence of legumain. It was crucial to perform this reaction before the release of the probe from the solid support to render the recognition sequence accessible to legumain. Preliminary results indicated that the deprotection was more efficient when performed prior to the attachment of the probe to the beads. The optimal reaction conditions include the catalyst $Pd(OAc)_2$, the water-soluble ligand trisodium 3,3′,3″-phosphanetriyltris(benzene-1-sulfonate (TPPTS), morpholine as a scavenger, and the addition of $MgCl_2$ to prevent the unprotected peptide from reacting with the catalyst.[57] The reaction was monitored by UHPLC-MS and full conversion was observed after 2 hours. It was necessary to include a washing step with a saturated aqueous Na_2SO_4 solution to remove excess of $MgCl_2$.

With the optimized conditions in hand, the procedure (Fig. 4B and Fig. S4, ESI†) with probe **11** was applied to the catch, enrichment, and release of the natural product pseudoephedrine (**2**). For the catching step, compound **2** was coupled to probe **11** with HATU and the protection groups were removed. The enrichment was performed by incubating the modified probe with magnetic beads to remove unreacted reagents and side products. The release from the solid support was then achieved with a 5 mM biotin solution. The natural product **2** was recovered in a 26% yield after treatment with legumain. We hypothesised that this recovery is mainly due to the efficacy of the enzymatic cleavage due, most likely, to having aspartic acid at position P1. To further demonstrate the chemoselectivity of our novel protocol, probe **11** was examined in a controlled complex matrix containing the following compounds at an equimolar concentration: serotonin (**1**), pseudoephedrine (**2**), 1-deoxynojirimycin, isopropanol, prop-2-en-1-ol, 4-methylbenzenethiol, histamine, acarbose, and vancomycin. We were glad to obtain **1** and **2** and 1-deoxynojirimycin as the underivatized natural products with a recovery yield of 9%, 3%, and <1%, respectively. Furthermore, the masses corresponding to the probe reacted with histamine, and acarbose were also observed after the release from the solid support.

In addition, we tested our protocol with a sample collected from cyanobacteria mats detected in Neuchâtel, Switzerland. The biomass was extracted and submitted to the catch-enrich-release strategy using probe **11**. The extract and released product were analysed by UHPLC-HRMS/HRMS to identify compounds present in both samples. A comparison with a cyanobacteria metabolites database (CyanMetDB)[58] led to the identification of two potent neurotoxins: anatoxin-a (**3**) and dihydroanatoxin-a (Table S1 and Fig. S5, ESI†). The structure of both compounds was confirmed by HRMS/HRMS analysis and comparison with analytical standard (Fig. S6 and S7, ESI†).[59]

In conclusion, we developed the first catch-enrich-release approach that results underivatized amine-containing compounds as products. The robustness of the strategy was demonstrated with the natural products serotonin (**1**), pseudoephedrine (**2**), 1-deoxynojirimycin, anatoxin-a (**3**), and dihydroanatoxin-a. Furthermore, the optimized probe **11** is quickly accessible *via* SPPS and can be readily used to improve the discovery and identification of amine-containing natural products.

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Conflicts of interest

There are no conflicts to declare.

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