Tetrahedron Letters 59 (2018) 3084–3087

Contents lists available at ScienceDirect

Tetrahedron Letters

journal homepage: www.elsevier.com/locate/tetlet

Preparation of new halogenated diphenyl pyrazine analogs in *Escherichia coli* by a mono-module fungal nonribosomal peptide synthetase from *Penicillium herquei*

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ARTICLE INFO

Article history:
Received 10 May 2018
Revised 20 June 2018
Accepted 28 June 2018
Available online 2 July 2018

Keywords:
Diphenyl pyrazine
Biosynthesis
NRPS
Tyrosine recognition
Enzyme promiscuity

ABSTRACT

Pyrazines are important structures widely found in many known drugs. The biological approaches for their synthesis were poorly applied. Herein, microbial production of several halogenated diphenyl pyrazines is reported. These compounds are accumulated via feeding corresponding precursor analogs to *Escherichia coli* expressing a fungal non-ribosomal peptide synthetase HqlA. Substrate specificity of HqlA was also determined by comparing substrate incorporation efficiencies. HqlA requires a C4-hydroxyl in the substrate and can tolerate certain degrees of size change on the substitution at the carbon next to the hydroxyl group.

Introduction

Pyrazines are an important class of heterocyclic compounds containing the 1,4-diazine core. The pyrazine derivatives not only exhibit characteristic reactivities, such as acting as bridging ligand to combine two molecules, but also possess ideal pharmacological activities [1,2]. Many of them have been developed into clinical drugs, for example, the proteasome inhibitor Bortezomib, antidiabetic agent Glipizide, anti-tubercular agents Morinamide and Pyrazinamide (Fig. 1) [3–6]. Moreover, pyrazine derivatives are used as flavoring additives in food [7]. The synthesis of pyrazines has been studied for years. Although a number of chemical routes for substituted pyrazines have been designed, the biological approaches were poorly applied.

Biosynthesis of 2,5-substituted pyrazines had been proposed to use amino acids as building blocks and corresponding diketopiperazines as intermediates. However, research from Wittmann et al. on biosynthesis of some highly substituted pyrazines in *Corynebacterium glutamicum* supported that those pyrazines are synthesized from intermolecular condensation of alpha-amino-ketones [8]. Feeding experiments by Schulz and co-workers confirmed amino acid as precursor and excluded involvement of diketopiperazine as intermediate, they also proposed that pyrazines are probably dimerization products from the corresponding α-minocarbonyl intermediates [9].

A few enzymatic/genetic studies had been reported on synthesis of pyrazines. Tang et al. produced a series of thiopyrazines by using a truncated nonribosomal peptide synthetase (NRPS325) module from a PKS-NRPS hybrid protein ATEG00325 [10], which is involved in the biosynthesis of isoflavipucine and dihydroisoflavipucine from *Aspergillus terreus* [11]. As another example, Keller et al. correlated the formation of diphenylpyrazines with the redundant clusters including the NRPS genes *lnaA* and *lnbA* in *Aspergillus flavus* [12]. In the previous study for biosynthesis of piperazine alkaloid Herquline A in *Penicillium herquei*, we found that a NRPS enzyme HqlA could efficiently produce the diphenyl pyrazine **2** [13].

HqlA is a single module NRPS-like protein consisting of an adenylation domain (A), a thiolation domain (T) and a reductase domain (R) (Fig. 2) [13]. Unlike a typical NRPS, HqlA lacks the key condensation domain (C) that is responsible for the peptide bond formation. However, HqlA still follows the same thioester-tethered mechanism as in NRPS mediated biosynthesis, where each module contributes a single amino acid to the assembling intermediate tethered on the carrier proteins [14–16]. Specifically, the A domain in HqlA recognizes and activates L-tyrosine to from the tyrosyl-T thioester intermediate, then the R domain reduces the intermediate into the corresponding amino aldehyde.

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https://doi.org/10.1016/j.tetlet.2018.06.065
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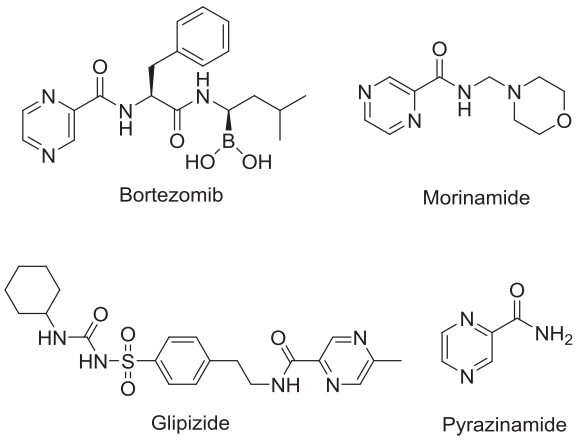

Fig. 1. Examples of pyrazine-containing drugs.

Dimerization between two aldehydes yields the diphenyl pyrazine **2** (Fig. 2). In recent years, efforts were performed for the manipulations of recombinant NRPSs to produce novel peptides [17,18]. For HqlA, elucidation of the substrate promiscuity of this protein is the first step to manipulate it for the biocombinatorial synthesis of substituted pyrazines.

Results

We overexpressed the recombinant *P. herquei* HqlA in its *holo* form with a C-terminal His_6-tag in *Escherichia coli* BAP1 cells [19]. Since most NRPSs show low enzymatic efficiencies on *in vitro* reconstituted assays and only trace amounts of products can be detected, we chose a whole-cell biotransformation approach to characterize the substrate promiscuity of HqlA. The *in vivo* biotransformation of *Escherichia coli* BAP1 cells harboring *hqlA* was performed with different substrates at 16 °C for 2 days (Fig. 3). Biotransformation by *E. coli* BAP1 cells with the empty vector pCDFDuet-1 was used as negative controls.

The first group of substrates was O-methyl-L-tyrosine (**9**) and DL-m-tyrosine (**10**). Since L-tyrosine (**1**) is a proteinogenic amino acid synthesized in *E. coli*, the corresponding product **2** was also detected in the biotransformation. However, when the hydroxyl group at C4 was replaced with a methoxy group or shifted to C3, no other product was detected, indicating that there is probably a key hydrogen binding between the C4-OH and the enzyme, therefore the presence of C4-OH is necessary for the substrate recognition in HqlA.

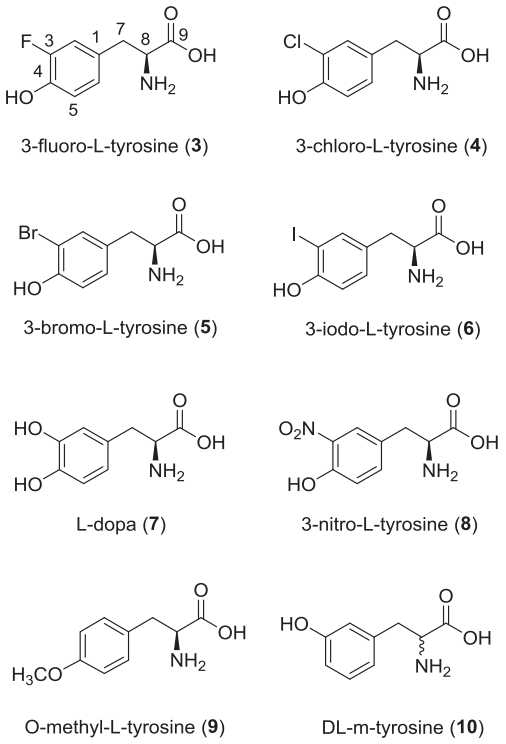

Fig. 3. Tyrosine derivatives investigated in this study.

To further probe the substrate specificity of HqlA, we tested six tyrosine derivatives with C4-OH and different C3 substitution groups, including 3-fluoro-L-tyrosine (**3**), 3-chloro-L-tyrosine (**4**), 3-bromo-L-tyrosine (**5**), 3-iodo-L-tyrosine (**6**), L-dopa (**7**) and 3-nitro-L-tyrosine (**8**). The results indicated that the substrate acceptance in HqlA is related to the size of the substitution groups at C3. HqlA utilized tyrosine derivatives with smaller groups (F-, Cl- or Br-, *i.e.* compounds **3**–**5**) on C3, but not those with larger substitution groups (I- and NO_2–, *i.e.* compounds **6** and **8**). Detailed inspection of the HPLC chromatograms for biotransformation of **3**–**5** revealed that two new products were detected in the extracts (Fig. 4). Reduced total conversion yield was found for **3**, **4** and **5** in corresponding to the larger C3– substitution group. Specific product yield from **3**, **4** and **5** by HqlA was 3.7, 0.9 and 0.4, respectively, which are represented by the ratio between the amount of the new compounds to the amount of **2** in the background (Fig. 4). These results suggest the inverse relation of the sizes of the C3 substitution groups to the HqlA activities. In the case of

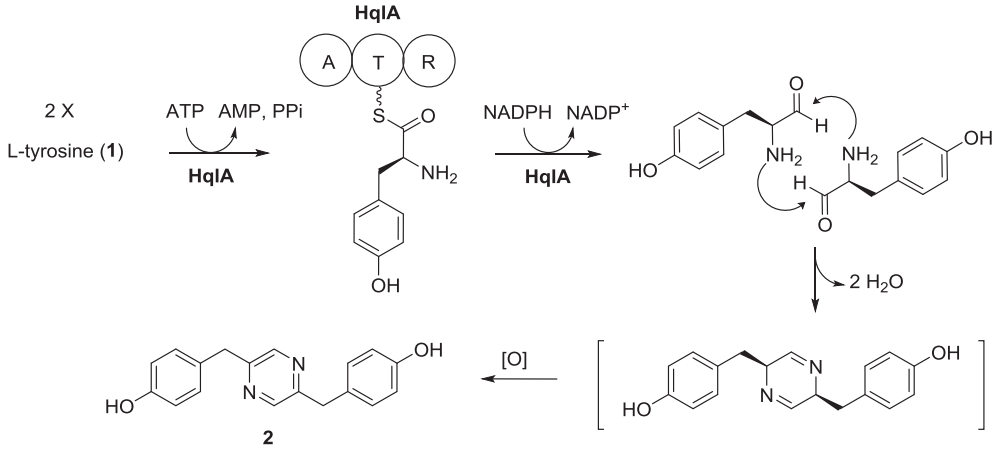

Fig. 2. Reaction of HqlA by using the natural substrate L-tyrosine.