ChemPubSoc Europe

DOI: 10.1002/cbic.201402686

CHEMBIOCHEM Full Papers

A Mutasynthesis Approach with a *Penicillium chrysogenum* Δ*roqA* Strain Yields New Roquefortine D Analogues

Kahina Ouchaou,[a] Florian Maire,[b] Oleksandr Salo,[c] Hazrat Ali,[c] Thomas Hankemeier,[b] Gijsbert A. van der Marel,[a] Dmitri V. Filippov,[a] Roel A. L. Bovenberg,[d, e] Rob J. Vreeken,[b] Arnold J. M. Driessen,*[c] and Herman S. Overkleeft*[a]

Penicillium chrysogenum, which lacks the *roqA* gene, processes synthetic, exogenously added histidyltryptophanyldiketopiperazine (HTD) to yield a set of roquefortine-based secondary metabolites also produced by the wild-type strain. Feeding a number of synthetic HTD analogues to the Δ*roqA* strain gives rise to the biosynthesis of a number of new roquefortine D derivatives, depending on the nature of the synthetic HTD added. Besides delivering semisynthetic roquefortine analogues, the mutasynthesis studies presented here also shed light on the substrate preferences and molecular mechanisms employed by the roquefortine C/D biosynthesis gene cluster, knowledge that may be tapped for the future development of more complex semisynthetic roquefortine-based secondary metabolites.

Introduction

Roquefortines C and D (Scheme 1) and secondary metabolites derived from them are produced by many different *Penicillium* species, including *Penicillium chrysogenum*.[1] They are members of the broad family of prenylated indole alkaloids[2,3] and are derived from histidyltryptophanyldiketopiperazine (HTD, Scheme 2) as the distinguishing precursor. Roquefortines and their derivatives are of interest because of their structural complexity, their biosynthesis, and their biological activity. Roquefortine C was identified to have neurotoxic activity in mice and is bacteriostatic against a number of Gram-positive bacteria.[4–6] A downstream product derived from roquefortine C is neoxaline, also a compound with attributed antimicrobial activity.[7] Our work on roquefortines to date has focused on unraveling their biosynthesis pathway.

We recently reported that roquefortines C and D are produced in *P. chrysogenum* by a parallel biosynthesis pathway.[8,9] In the first step, the common precursor, HTD, is synthesized by the nonribosomal peptide synthetase RoqA. HTD is subsequently transformed in a series of steps into roquefortines and from these into a number of complex secondary metabolites.

The biosynthesis of the roquefortine indole alkaloids involves two gene clusters.[8,10] RoqA is a nonribosomal peptide synthetase (NRPS) cluster that accepts and activates L-histidine and L-tryptophan and condenses these to produce HTD. This diketopiperazine is accepted as substrate by a further set of enzymes including the dehydrogenase RoqR and the dimethylallyltryptophan synthase RoqD. These, in concert with a number of other gene products, are responsible for the biosynthesis of roquefortines C and D and their downstream secondary metabolites.

The parallel action of RoqA and RoqR/RoqD invites a strategy in which roquefortine analogues are produced through a mutational biosynthesis—or mutasynthesis—approach.[11,12] In mutasynthesis a gene (or gene cluster) responsible for the production of a key secondary metabolite precursor is eliminated, and the resulting mutant strain is supplemented with a synthetic, modified version of this precursor in the hope that this will be taken up and processed to deliver new analogues of the original secondary metabolite. Mutasynthesis is considered to have the advantage over classical precursor-directed biosynthesis strategies in that competition between endogenous substrate and added synthetic substrate is eliminated.[11] On the downside, engineering NRPS or polyketide synthase (PKS) gene clusters to yield a desired mutant is high-risk in that the secondary

[a] *Dr. K. Ouchaou, Prof. Dr. G. A. van der Marel, Dr. D. V. Filippov, Prof. Dr. H. S. Overkleeft*
Leiden Institute of Chemistry, Department of Bio-organic Synthesis
Leiden University
Einsteinweg 55, 2333 CC Leiden (The Netherlands)
E-mail: h.s.overkleeft@chem.leidenuniv.nl

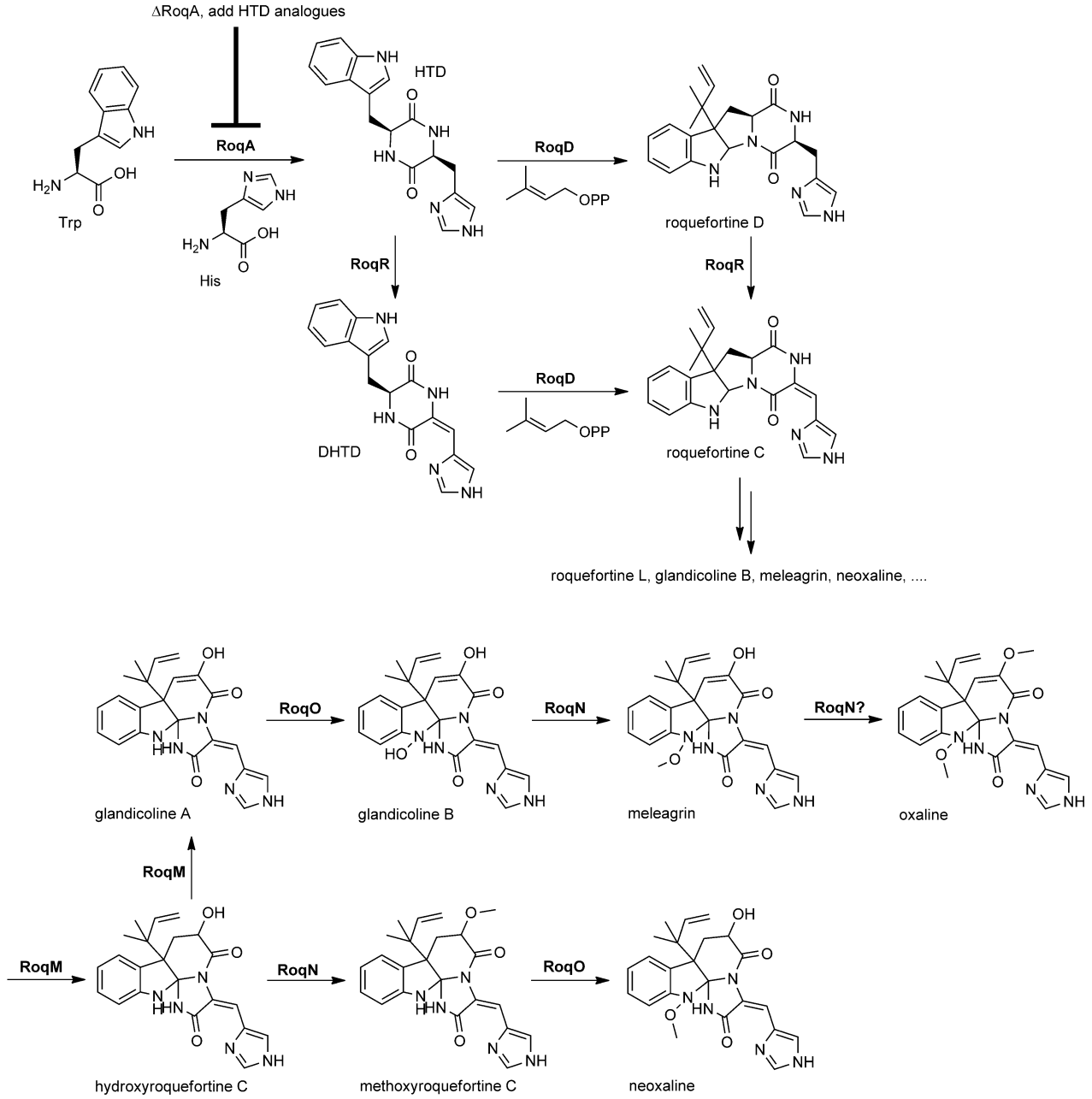
[b] *Dr. F. Maire, Prof. Dr. T. Hankemeier, Dr. R. J. Vreeken*
Division of Analytical Biosciences
Leiden Academic Centre for Drug Research
Einsteinweg 55, 2333 CC Leiden (The Netherlands)

[c] *O. Salo, Dr. H. Ali, Prof. Dr. A. J. M. Driessen*
Department of Molecular Microbiology
Groningen Biomolecular Sciences and Biotechnology Institute
Zernike Institute for Advanced Materials, University of Groningen
Nijenborgh 7, 9747 AG Groningen (The Netherlands)
E-mail: a.j.m.driessen@rug.nl

[d] *Prof. Dr. R. A. L. Bovenberg*
DSM Biotechnology Center
Alexander Fleminglaan 1, 2613 AX Delft (The Netherlands)

[e] *Prof. Dr. R. A. L. Bovenberg*
Department of Synthetic Biology and Cell Engineering
Groningen Biomolecular Sciences and Biotechnology Institute
University of Groningen
Nijenborgh 7, 9747 AG Groningen (The Netherlands)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201402686.



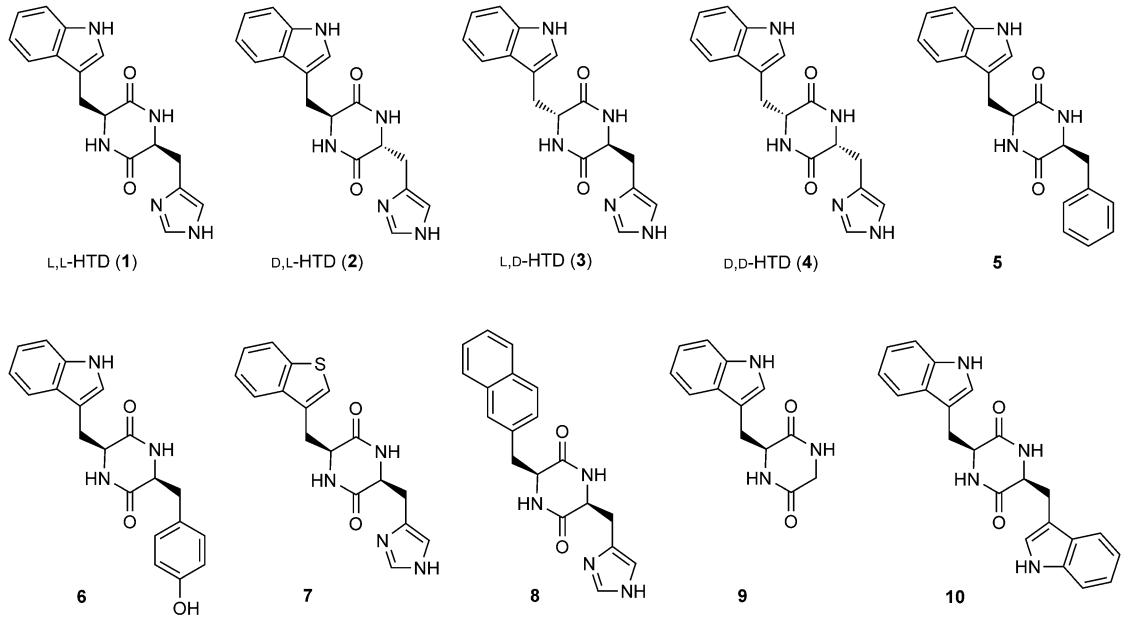
Scheme 1. Biosynthetic pathway to roquefortines C and D in *P. chrysogenum* and mutasynthesis strategy reported here (PP = pyrophosphate).

metabolite biosynthesis machinery as a whole is compromised.[12]

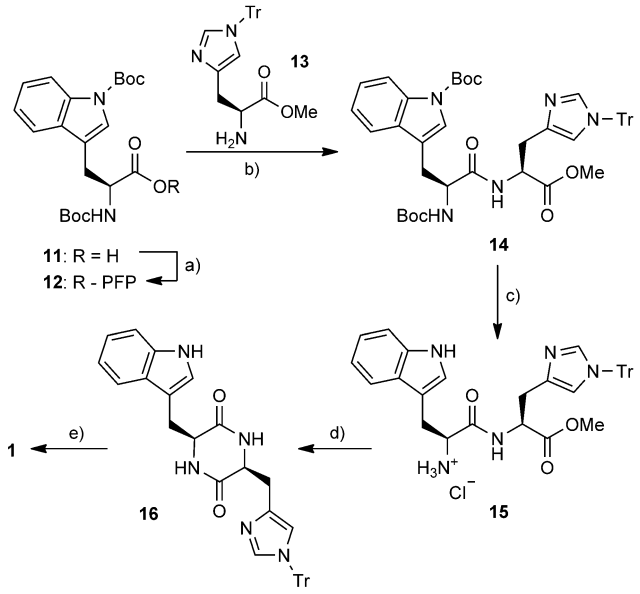
We reasoned that because of its parallel action of RoqA and RoqR/D the roquefortine biosynthesis pathway would not be subject to this caveat. On the basis of this assumption, we set out to apply our *P. chrysogenum* strain in which we had genetically deleted the *roqA* gene (*P. chrysogenum* Δ*roqA*), as described in ref. [8], to reconstitute roquefortine biosynthesis by external addition to the growth media of HTD. Here we show our results in this undertaking, as well as the fate of a set of HTD analogues, **2**–**10** (Scheme 2), in terms of roquefortine analogue mutasynthesis.

Results and Discussion

A number of synthesis strategies for the preparation of chiral, enantiopure diketopiperazines have appeared in the literature.[13–18] Perusal of these reveals that the preparation of L,L- or D,D-diketopiperazines—that is, the synthesis of diketopiperazines assembled from two L- or D-amino acids—is considerably more complicated than the construction of their L,D-configured counterparts. Thus, as the first research objective we investigated the synthesis of the natural roquefortine precursor L,L-HTD (**1**). The optimized route we arrived at, which is based on literature[17,19] precedent, is shown in Scheme 3.



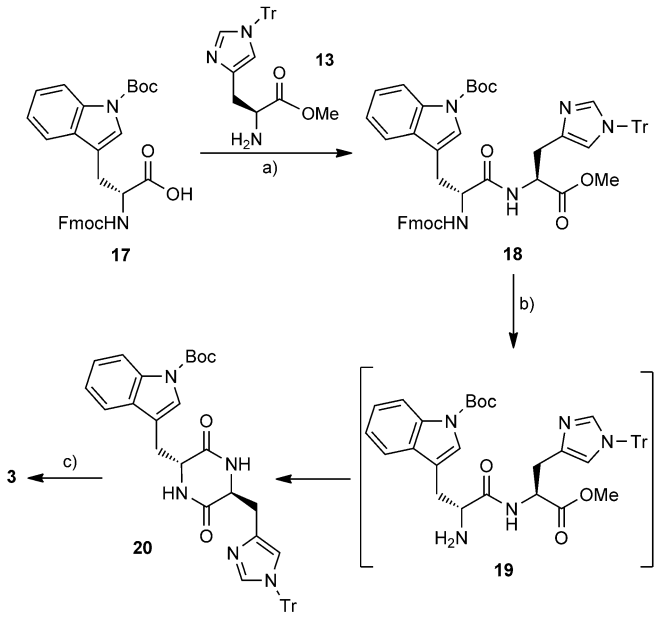
Scheme 2. L,L-HTD (**1**) and synthetic HTD isomers and analogues **2**–**10**, subjects of this mutasynthesis study.



Scheme 3. Synthesis of L,L-HTD (**1**). a) C_6F_5OH, EDC·HCl, CH_2Cl_2, RT, 12 h, 74%; b) NEt_3, CH_2Cl_2, RT, 12 h, 87%; c) TMSI, CH_3CN, 0 °C, 2 h; d) $NH_3{\cdot}H_2O$, MeOH, RT, 16 h, 45%; e) 20% TFA, 2.5% TIS, CH_3CN, 0 °C, 2 h, 99%.

In the first step, bis-Boc-protected tryptophan **11** was transformed into the corresponding pentafluorophenyl ester, which was condensed with partially protected histidine **13**. The resulting fully protected dipeptide **14** was treated with trimethylsilyl iodide (TMSI) under conditions reported in the literature[19] to deblock the tryptophan secondary amine selectively. Although the yield in this step was only moderate, structurally and enantiomerically pure H_2N dipeptide **15** was obtained in this way. Application of other acidic conditions to obtain **15** proved abortive, and we were able to prepare sufficient quantities of the desired intermediate to continue our synthesis by using TMSI. Treatment of in-situ-generated H_2N dipeptide **15** with aqueous ammonia yielded monotrityl-protected HTD **16**, which was treated with trifluoroacetic acid in dichloromethane in the presence of triisopropylsilane (TIS) as a cation scavenger to yield target compound **1**.

An alternative route to diketopiperazines more commonly applied in the literature[20–22] is depicted in Scheme 4 for the synthesis of L,D-HTD (**3**). This route is based on condensation of an Fmoc-protected α-amino acid (here Fmoc-(Boc)-D-trypto-



Scheme 4. Synthesis of L,D-HTD (**3**). a) HATU, Et_3N, CH_3CN, RT, 12 h, 99%; b) 50% piperidine in DMF, RT, 2 h, 62%; c) 20% TFA, 2.5% TIS, CH_3CN, 0 °C, 2 h, 96%.

phan (**17**)) with a second α-amino acid protected as the methyl ester (here L-histidine derivative **13**). In this way, fully protected dipeptide **18** is obtained uneventfully. The difference between the two routes is in the next stage: whereas in the former example deprotection of the secondary amine proceeded under (Lewis) acidic conditions, in the current case the Fmoc group is removed under basic (piperidine in DMF) conditions. The advantage in terms of efficiency is that, upon the Fmoc removal, the liberated basic amine in **19** reacts without further manipulation with the methyl ester to provide protected diketopiperazine **20** in good yield. A disadvantage of this method—at least according to literature reports—is that the basic conditions applied can give rise to deprotonation of one of the two α-carbons and thus epimerization and erosion of enantiomeric purity. Nevertheless, after removal of the Boc/trityl protective groups in **20** and HPLC purification we were able to obtain L,D-HTD (**3**) in good yield and enantiomeric purity. We thus have two routes at our disposal, and these combined allowed us to prepare HTD analogues **1**, **2**, **5**, and **6** (by the TMSI route depicted in Scheme 3) as well as **3**, **4**, **7**, and **8** (by the Fmoc peptide chemistry route depicted in Scheme 4). HTD analogues **9** and **10** in turn were obtained from a commercial source.

With L,L-HTD (**1**) and its configurational isomers (compounds **2**–**4**) and structural analogues (compounds **5**–**10**) to hand, we set out to study their use in the mutasynthesis of roquefortine analogues by *P. chrysogenum* Δ*roqA*. Thus, after 4 days of growth of this strain, L,L-HTD was added at 200 μg mL^{-1} (0.6 mM) final concentration, and growth was continued up to days 5 or 7. Next, the metabolites produced were analyzed in samples taken from both 5- and 7-day culture broths after removal of the cells by filtration, previous work having shown that roquefortine-related secondary metabolites can be readily extracted from the medium.[8,9] As shown in Figure 1 B, after 7 days of fermentation the *P. chrysogenum* Δ*roqA* strain, previously prepared by us,[8] had produced neither L,L-HTD nor any of its derived secondary metabolites. Supplementation with synthetic L,L-HTD (**1**), in contrast, restored the roquefortine metabolic pathway (Figure 1 C).

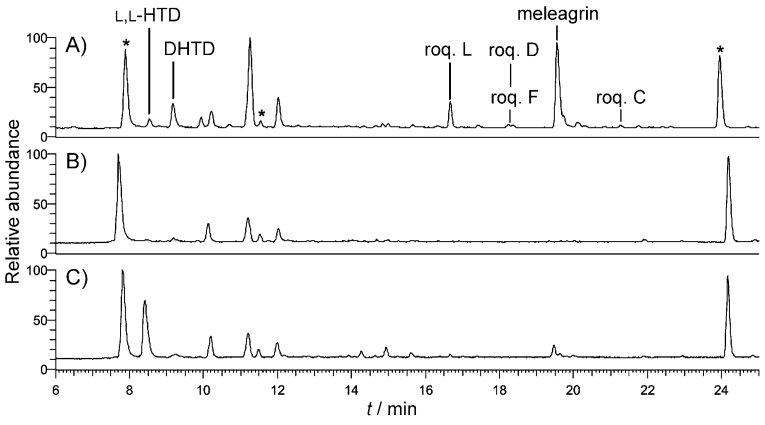


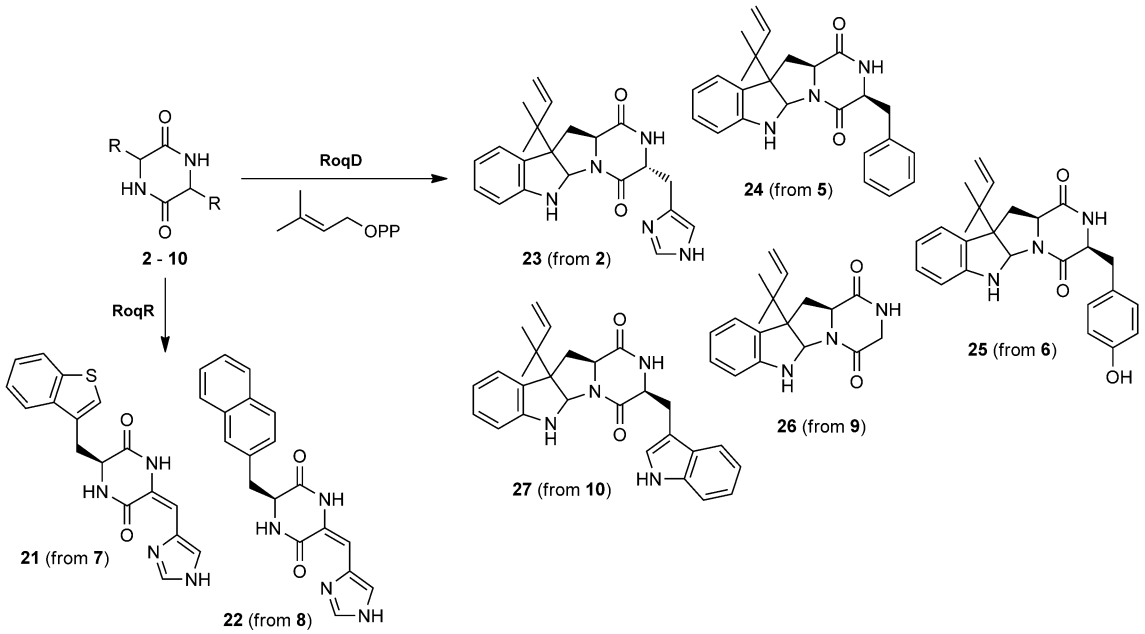
Figure 1. LC-MS chromatograms (total ion current): A) of production of roquefortines by wild-type *P. chrysogenum*, and of secondary metabolite production by *P. chrysogenum* Δ*roqA* B) in the absence and C) in the presence of L,L-HTD at 200 μg mL^{-1} after 7 days of fermentation (roq. roquefortine, * internal standard).

The outcome of feeding experiments in which the *P. chrysogenum* Δ*roqA* strain was grown with each of the configurational isomers and structural HTD analogues **2**–**10** is depicted in Scheme 5 (see the Supporting Information for LC-MS traces of these feeding experiments). Growth media were extracted and analyzed for roquefortine analogue content by LC-MS essentially as done for the feeding experiment with synthetic L,L-HTD (**1**).

Exact masses of putative roquefortine analogue metabolites were calculated and compared against the list of ions highlighted during the data processing of the LC-MS chromatograms (i.e., ions present in supplemented *P. chrysogenum* Δ*roqA* cultures and absent in *P. chrysogenum* Δ*roqA* control culture). It should be noted that no NMR experiment has been performed on the roquefortine analogue metabolites. Because the identifications are based on accurate masses only, isomers of the structures shown in Scheme 5 cannot be ruled out completely. A first observation we made is that neither of the HTD analogues made it through the biosynthesis pathways to produce roquefortine C analogues. At least, if such analogues—or any of the secondary metabolites derived from roquefortine C—had been produced, this had occurred in quantities below our limit of detection. On the positive side, some HTD analogues proved to be acceptable substrates for RoqR (the dehydrogenase activity) and some were accepted by RoqD (the dimethylallyltryptophan synthase activity), whereas configurational isomers **3** and **4** proved metabolically inert.

Closer perusal of the obtained data allows for some interesting observations. With respect to the configurational isomer set **1**–**4**, L,D-HTD (**3**) and D,D-HTD (**4**) proved to be unacceptable substrates for both RoqD and RoqR, and from this we can conclude that the L stereochemistry of the tryptophan residue is essential. This is underscored by the observation that D,L-HTD (**2**) is accepted by RoqD to produce the roquefortine C analogue **23** (Figure 2). Neither **23** nor **2** is dehydrogenated, and so the L configuration of the histidine residue is essential for RoqR as well.

Structural analogues **5**–**10** all have the appropriate stereochemistry for both RoqD and RoqR, and acceptance by either of the two therefore relies on their structural and functional features. RoqR-mediated dehydrogenation proceeds with **7** (to produce **21**, Figure 3) and with **8** (to produce **22**), in other words exclusively with those HTD analogues containing L-histidine as one of the two α-amino acids incorporated. RoqD in turn is lenient with regard to the nature of the L-histidine analogue but accepts, out of the series of HTD analogues screened in this study, only those residues featuring an L-tryptophan moiety. In this way, roquefortine C analogues **24** (from **5**), **25** (from **6**), **26** (from **9**), and **27** (from **10**) are produced. The fact that none of these metabolites is further processed to roquefortine C analogues can also be seen as a positive outcome in that in this way roquefortine D analogues—in themselves interesting compounds in terms of their structural complexity—can be readily prepared. Somewhat surprising is the finding that HTD ana-



Scheme 5. DHTD/roquefortine D analogues produced by mutasynthesis of *P. chrysogenum* Δ*roqA* broth supplemented with HTD isomers and analogues **2**–**10**.

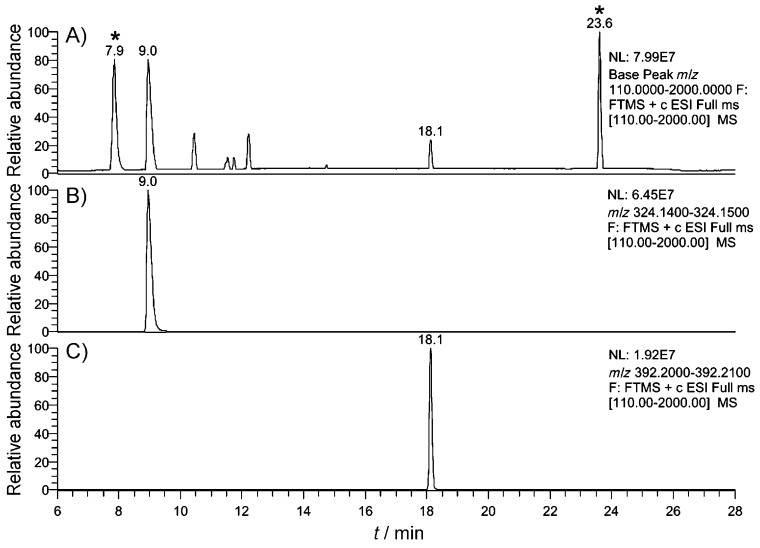


Figure 2. LC-MS analysis of culture broth of *P. chrysogenum* Δ*roqA* 72 h after addition of **2**. A) Full chromatogram (* internal standard). B) Extracted ion chromatogram of **2**. C) Extracted ion chromatogram of **23**. The analysis was performed with the LTQ-Orbitrap, and an internal recalibration was performed after acquisition by use of the monoprotonated ion of **2**.

logues **7** and **8**, featuring a benzothiophene and a naphthyl moiety, respectively, as indole analogues, are not acceptable RoqD substrates. The indole nitrogen appears to be crucial in the electrophilic aromatic substitution that comprises the first step in the RoqD-catalyzed indole prenylation process. In view of this result it would be of interest to investigate the fates of benzofuran-based HTD analogues or of analogues of **7** and **8** in which the benzothiophene/naphthyl moieties are modified to bear electron-donating substituents.

Conclusions

In conclusion, this work demonstrates that the roquefortine biosynthesis pathway is amenable to mutasynthesis studies to deliver new, semisynthetic DHTD/roquefortine D analogues. From an engineering point of view, it can be predicted from the branched nature of the roquefortine biosynthesis pathway that the deletion of the *roqA* gene should yield a strain in which the complete roquefortine biosynthesis pathway can be rescued through supplementation with synthetic L,L-HTD. The two enzymes immediately downstream of HTD biosynthesis, RoqD and RoqR, appear to be selective for their corresponding amino acids (L-Trp and L-His, respectively), but not so much for the second amino acid that completes the diketopiperazine ring. Thus, the small set of HTD analogues assessed here has already delivered some interesting semisynthetic secondary metabolites, in particular roquefortine D analogues **23**–**27**. Further modifications on the HTD core, such as the benzofuran tryptophan analogue mentioned above, might yield semisynthetic secondary metabolites with structures resembling those found further downstream in the roquefortine biosynthesis pathway. Here it should be mentioned that our approach is biased towards the detection of modified metabolites that are exported into the medium. We cannot exclude the possibility that additional metabolites are produced from our HTD analogues but are not detected because they are not exported. Finally, numerous indole alkaloid secondary metabolites found in nature are derived from diketopiperazines featuring L-tryptophan together with α-amino acids other than histidine.[23] Generation of the corresponding diketopiperazine synthase deletion mutants

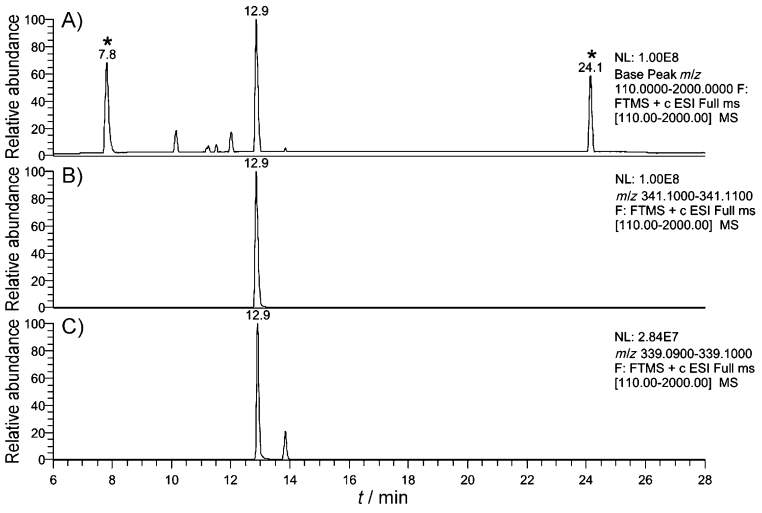


Figure 3. LC-MS analysis of culture broth of *P. chrysogenum* Δ*roqA* 72 h after addition of **7**. A) Full chromatogram (* internal standard). B) Extracted ion chromatogram of **7**. C) Extracted ion chromatogram of **21**. The analysis was performed with the LTQ-Orbitrap, and an internal recalibration was performed after acquisition by use of the monoprotonated ion of **7**.

should allow mutasynthesis studies related to those presented here, especially in the light of the diketopiperazine synthesis procedures we have developed, and thus enable the synthesis of both configurational and structural diketopiperazine analogues.

Experimental Section

General: All reagents were commercial grade and were used as received unless indicated otherwise. Dichloromethane was distilled over phosphorus pentoxide. DMF, MeCN, MeOH, piperidine, and NEt_3 were stored over molecular sieves (4 Å). Reactions were monitored by TLC (DC-Alufolien, Merck, Kieselgel 60, F254) with detection variously by UV absorption (254 nm), by spraying with a solution of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (25 g L^{-1}) and $(NH_4)_4Ce(SO_4)_4{\cdot}2H_2O$ (10 g L^{-1}) in sulfuric acid (10%) followed by charring at ≈150 °C, or by spraying with an aqueous solution of $KMnO_4$ (20%) and K_2CO_3 (10%). Column chromatography was performed on silica gel (Screening Devices, 0.040–0.063 nm). LC/MS analysis was performed with a LCQ Advantage Max (Thermo Finnegan) instrument with a Gemini C18 column (Phenomenex). The solvents used were H_2O (A), MeCN (B), and aq. TFA (1.0%, C). ^1H and ^{13}C APT-NMR spectra were recorded with Bruker AV 400 (400/100 MHz) instruments with a pulsed field gradient accessory. Chemical shifts (δ) are given in ppm relative to tetramethylsilane as internal standard. Coupling constants are given in Hz. All ^{13}C-APT spectra presented are proton-decoupled.

(L)-BocTrp(Boc)pentafluorophenyl ester (12): Pentafluorobenzophenol (0.456 g, 2.48, 2.0 equiv) was added under argon to a solution of *N*-Boc-*N'*-Boc-L-tryptophan (0.5 g, 1.24 mmol, 1.0 equiv) in freshly distilled CH_2Cl_2 (6.5 mL, 0.2 M), followed by EDC·HCl (0.474 g, 2.48 mmol, 2.0 equiv). The mixture was stirred at room temperature overnight. The reaction was quenched with a solution of HCl (1 M), and the mixture was extracted with Et_2O. The organic layer was washed with brine and dried over $MgSO_4$, and the solvent was removed. The mixture was purified on silica (pentane/EtOAc 100:0→97:3) to give the corresponding compound as a white solid (0.522 g, 74%). ^1H NMR (400 MHz, $CDCl_3$): δ = 8.21–8.12 (br d, 1H), 7.57 (d, J = 7.6 Hz, 1H), 7.52 (s, 1H), 7.35 (m, 1H), 7.27 (m, 1H), 5.05 (m, 1H), 3.45 (m, 1H), 3.35 (m, 1H), 1.67 (s, 9H), 1.44 ppm (s, 9H).

Dipeptide Boc-Trp(Boc)-His(Tr) methyl ester (14): A solution of L-BocTrp(Boc)OC_6F_5 (0.52 g, 0.9 mmol, 1.0 equiv) in freshly distilled CH_2Cl_2 (5 mL) was added dropwise under argon to a solution of L-His(Tr)OMe (0.45 g, 1.0 mmol, 1.1 equiv) and NEt_3 (0.14 mL, 1.1 equiv) in freshly distilled CH_2Cl_2 (5 mL). The reaction mixture was stirred overnight at room temperature. The solvent was removed, and the mixture was purified on silica (MeOH in CH_2Cl_2, 1%) to afford the expected compound as an amorphous white solid (0.63 g, 87%). ^1H NMR (400 MHz, $CDCl_3$): δ = 8.12–8.01 (br s, 1H), 7.60 (d, J = 6.8 Hz, 1H), 7.46 (s, 1H), 7.38–7.15 (m, 12H), 7.11–7.03 (m, 6H), 6.43 (s, 1H), 4.78–4.71 (m, 1H), 4.59–4.48 (m, 1H), 3.55 (s, 3H), 3.38–3.10 (m, 2H; AB), 3.05–2.84 (m, 2H; AB), 1.64 (s, 9H), 1.36 ppm (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.17–171.09, 165.64, 155.40, 149.55, 141.16, 135.51, 130.53, 129.78, 128.54, 124.37, 122.54, 119.86, 119.26, 116.07, 115.14, 83.43, 79.75, 54.79, 52.77, 52.58, 29.05, 28.31–28.23 ppm; LC-MS: *m/z*: 798.13 $[M+H]^+$, 1594.73 $[2M]^+$.

***Cyclo*-L-Trp-L-His(Tr) (16)**: TMSI (150 μL, 6.0 equiv) was added at 0 °C to a solution of compound **15** (0.135 g, 0.17 mmol, 1.0 equiv) in dry CH_3CN (6 mL). The reaction mixture was allowed to warm to room temperature over 3 h. The reaction was quenched with a solution of aqueous saturated $NaHCO_3$, and the mixture was extracted with CH_2Cl_2. The organic layer was washed with water and brine and then dried over $MgSO_4$. After removal of the solvent, the crude product was dissolved in dry MeOH (6 mL), and aq. NH_3. (0.6 mL) was added. The solution mixture was then stirred overnight and concentrated under reduced pressure. Purification by column chromatography (MeOH in CH_2Cl_2, 4%) yielded the purified compound as an amorphous beige solid (0.043 g, 45%). ^1H NMR (400 MHz, $CDCl_3$): δ = 8.78 (s, 1H), 7.54 (d, J = 8.0 Hz, 1H), 7.35–7.14 (m, 14H), 7.09–6.80 (m, 9H), 6.15 (s, 1H), 4.30–4.20 (br s, 1H), 4.15–4.11 (br d, 1H), 3.52–3.46 (br dd, 1H; AB), 3.18–3.12 (br dd, 1H; AB), 2.99–2.95 (br dd, 1H; AB), 1.86–1.82 ppm (br t, 1H; AB); ^{13}C NMR (100, MHz, $CDCl_3$): δ = 168.02, 167.30, 142.27, 138.92, 136.28, 136.21, 129.80, 128.23, 127.25, 124.49, 122.24, 120.07, 119.82, 118.97, 111.36, 109.14, 75.44, 55.13, 54.85, 31.67, 30.03 ppm; LC/MS: 565.93 $[M+H]^+$, 1130.80 $[2M]^+$.

L,L-HTD (1): A solution of compound **17** (0.043 g, 0.07 mmol) in dry CH_2Cl_2 (6 mL) was cooled to 0 °C. Then TFA (20%) and TIS (2.5%) were added. The reaction mixture was concentrated in the presence of toluene and then stirred for 2 h. Purification by column chromatography (10% MeOH in EtOAc) yielded the compound as a white solid (0.023 g, 97%). Spectroscopic data were in accordance with known literature values.[24] $[\alpha]_D^{20} = -42$ (c = 4.06, H_2O); 1H NMR (400 MHz, D_2O): δ = 8.26 (d, J = 1.6 Hz, 1H), 7.60 (d, J = 8.0 Hz, 1H), 7.49 (d, J = 8.0 Hz, 1H), 7.28 (dd, J = 8.0, 1.2 Hz, 1H), 7.23–7.17 (m, 2H), 5.90 (d, J = 1.6 Hz, 1H), 4.46 (dd, J = 3.2, 1.2 Hz, 1H), 4.02 (dd, J = 4.8, 1.2 Hz, 1H), 3.46 (dd, J = 14.8, 3.2 Hz, 1H; AB), 3.13 (dd, J = 14.8, 4.8 Hz, 1H; AB), 2.32 (dd, J = 14.8, 4.4 Hz, 1H; AB), 1.37 ppm (dd, J = 14.8, 8.8 Hz, 1H; AB).); ^{13}C NMR (100, MHz, D_2O): δ = 169.68, 167.67, 135.85, 133.31, 127.37, 126.71, 125.70, 122.06, 119.72, 119.01, 117.35, 112.06, 107.62, 55.69, 53.09, 28.55, 28.30 ppm; HRMS: *m/z* calcd for $C_{17}H_{17}N_5O_2+H^+$: 324.1461 $[M+H]^+$; found: 324.1452.

Dipeptide Boc-Trp(Boc)-D-His(Tr) methyl ester (28): Applying the same procedure as described above for compound **15** yielded the title compound as an amorphous beige solid (0.38 g, 99%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.10–8.05 (brs, 1H), 7.85–7.80 (brs, 1H; NH), 7.58 (d, J = 7.6 Hz, 1H), 7.47–7.17 (m, 13H), 7.09–7.07 (m, 6H), 6.59 (s, 1H), 5.40–5.30 (brs, 1H; NH), 4.72–4.62 (brs, 1H), 4.50–4.40 (brs, 1H), 3.56 (s, 3H), 3.21–2.90 (m, 4H; AB), 1.61 (s, 9H), 1.35 ppm (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.17–171.09, 165.64, 155.40, 149.54, 141.61, 135.03, 130.57, 128.68, 128.20, 124.33, 122.47, 119.87, 119.07, 115.58, 115.11, 83.39, 79.88, 54.56, 52.46, 52.24, 28.77, 28.23, 28.13 ppm; LC-MS: *m/z*: 798.13 $[M+H]^+$, 1594.73 $[2M]^+$.

***Cyclo*-L-Trp-D-His(Tr) (29)**: Applying the same procedure as described above for compound **17** yielded the title compound as an amorphous beige solid (0.15 g, 52%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.91–8.40 (brs, 1H; NH_{indole}), 7.54 (d, J = 8.0 Hz, 1H), 7.43–7.40 (brs, 1H; NH), 7.32–7.20 (m, 11H), 7.13–6.96 (m, 9H), 6.86–6.76 (brs, 1H; NH), 6.56 (s, 1H), 4.08–4.02 (brs, 1H), 3.75–3.69 (brs, 1H), 3.40–3.35 (brdd, 1H; AB), 3.18–3.02 (m, 2H; AB), 2.78–2.68 ppm (brdd, 1H; AB); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 168.20, 167.91, 142.24, 138.76, 136.45, 136.25, 129.74, 128.21, 127.06, 124.37, 122.25, 119.74, 118.76, 111.47, 109.10, 55.61, 54.75, 31.20, 30.45 ppm; LC-MS: *m/z*: 565.93 $[M+H]^+$, 1130.80 $[2M]^+$.

L,D-HTD (2): The procedure was the same as described above for compound **1**. Purification on silica followed by HPLC gave the title compound as a white solid (0.06 g, 73%). Spectroscopic data were in accordance with known literature values.[24] $[\alpha]_D^{20}$ = +18 (c = 3.28, H_2O); ^{1}H NMR (400 MHz, CD_3OD): δ = 8.00 (s, 1H), 7.58 (d, J = 8.0 Hz, 1H), 7.33 (d, J = 8.0 Hz, 1H), 7.11–7.05 (m, 2H), 7.00 (t, J = 8.0 Hz, 1H), 6.79 (s, 1H,), 4.02 (t, J = 4.0 Hz, 1H), 3.40 (dd, J = 4.0, 14.4 Hz, 1H; AB), 3.20 (dd, J = 4.0, 14.4 Hz, 1H; AB), 3.03 (t, J = 4.4 Hz, 1H), 2.94 (dd, J = 4.4, 15.2 Hz, 1H; AB), 2.80 ppm (dd, J = 4.4, 15.2 Hz, 1H; AB); ^{13}C NMR (100 MHz, CD_3OD): δ = 169.46, 169.35, 135.67, 131.79, 128.84, 126.05, 125.98, 122.60, 120.18, 119.75, 118.89, 112.25, 109.04, 57.41, 54.67, 30.94, 29.57 ppm; LC-MS: *m/z*: 324.07 $[M+H]^+$; HRMS: *m/z* calcd for $C_{17}H_{17}N_5O_2+H^+$: 324.1461 $[M+H]^+$; found: 324.1450.

Dipeptide Boc-Trp(Boc)-Phe methyl ester (30): Applying the same procedure as described above for compound **15** yielded the title compound as an amorphous white solid (0.29 g, 69%). Spectroscopic data were in accordance with known literature values.[25] ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.13 (d, J = 6.8 Hz, 1H), 7.59 (d, J = 7.6 Hz, 1H), 7.44 (s, 1H), 7.32 (t, J = 7.2 Hz, 1H), 7.23 (t, J = 7.2 Hz, 1H), 7.16 (m, 3H), 6.88 (m, 2H), 6.27 (d, J = 6.4 Hz, 1H; NH), 5.09 (brs, 1H; NH), 4.72 (m, 1H), 4.43 (m, 1H), 3.62 (s, 3H), 3.13 (m, 2H), 2.98 (m, 2H; AB), 1.64 (s, 9H), 1.41 ppm (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.27, 170.91, 135.65, 130.34, 129.27, 128.61, 127.22, 124.77, 124.52, 122.86, 119.19, 115.40, 83.76, 54.70, 53.14, 52.37, 38.05, 28.38, 28.31, 28.18 ppm.

***Cyclo*-L-Trp-L-Phe (5)**: Applying the same procedure as described above for compound **17** yielded the title compound as an amorphous beige solid (0.06 g, 36%). Spectroscopic data were in accordance with known literature values.[26,27] ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 10.89 (s, 1H; NH), 7.91 (brs, 1H; NH), 7.70 (brs, 1H; NH), 7.48 (d, J = 8.0 Hz, 1H), 7.31 (d, J = 8.0 Hz, 1H), 7.19–7.15 (m, 3H), 7.07 (t, J = 6.8 Hz, 1H), 7.00–6.95 (m, 2H), 6.71–6.69 (m, 2H), 3.97 (brs, 1H), 3.86 (brs, 1H), 2.80 (dd, J = 14.4 Hz, 4.0 Hz, 1H; AB), 2.54–2.43 (m, 2H; AB), 1.84 ppm (dd, J = 13.2 Hz, 6.8 Hz, 1H; AB); ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 166.85, 166.23, 136.54, 136.07, 129.73, 128.07, 127.55, 126.41, 124.45, 120.93, 118.79, 118.47, 111.37, 108.82, 55.64, 55.28, 39.95, 39.73 ppm; HRMS: *m/z* calcd for $C_{20}H_{19}O_2N_3+H^+$: 334.1556 > $[M+H]^+$; found: 334.1548.

Dipeptide Boc-Trp(Boc)-Tyr(OBn) methyl ester (31): Applying the same procedure as described above for compound **15** yielded the title compound as an amorphous white solid (0.38 g, 79%); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.14 (d, J = 8.0 Hz, 1H), 7.59 (d, J = 8.0 Hz, 1H), 7.46–7.22 (m, 8H), 6.78–6.74 (m, 4H), 6.25 (d, J = 6.0 Hz, 1H; NH), 5.12–5.05 (brs, 1H; NH), 4.98 (s, 2H), 4.72–4.67 (m, 1H), 4.44–4.38 (m, 1H), 3.62 (s, 3H), 3.23–3.06 (m, 2H; AB), 2.93 (d, J = 6.0 Hz, 2H; AB), 1.62 (s, 9H), 1.41 ppm (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.37, 170.87, 158.04, 137.10, 130.33, 128.71, 128.10, 127.90, 127.65, 124.79, 124.57, 122.88, 122, 84, 119.23, 118.32, 115.48, 115.42, 114.99, 83.78, 70.09, 54.70, 53.44, 52.37, 37.23, 28.42, 28.33, 28.24 ppm.

***Cyclo*-L-Trp-L-Tyr(OBn) (32)**: Applying the same procedure as described above for compound **17** yielded the title compound as an amorphous beige solid (0.10 g, 43%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 10.89 (s, 1H; NH), 7.87 (brs, 1H; NH), 7.66 (brs, 1H; NH), 7.48 (d, J = 8.0 Hz), 7.53–7.22 (m, 6H), 7.07 (t, J = 8.0 Hz, 1H), 7.01–6.93 (m, 2H), 6.82 (d, J = 8.4 Hz, 2H), 6.61 (d, J = 8.4 Hz, 2H), 5.01 (s, 2H), 4.01–3.96 (brs, 1H), 3.78–3.82 (brs, 1H), 2.80 (dd, J = 14.4, 4.4 Hz, 1H; AB), 2.54–2.50 (m, 1H; AB), 2.42 (dd, J = 13.6 Hz, 4.4 Hz, 1H; AB), 1.80 ppm (dd, J = 13.6 Hz, 7.2 Hz, 1H; AB); ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 166.82, 166.24, 157.09, 137.18, 136.07, 130.74, 128.62, 128.38, 127.71, 127.53, 127.44, 124.42, 120.91, 118.72, 118.42, 114.42, 111.34, 108.88, 69.12, 55.75, 55.23, 38.89, 29.88 ppm.

***Cyclo*-L-Trp-L-Tyr (6)**: Pd/C (0.08 g) was added to a solution of compound **34** (0.081 g, 0.18 mmol) in EtOH (15 mL). After flushing with H_2 (balloon, three times), the reaction mixture was stirred overnight under H_2. After having been filtered on celite and concentrated, the crude product was purified on silica (MeOH in CH_2Cl_2, 5%) to afford the purified compound as a white solid (0.028 g, 45%). Spectroscopic data were in accordance with known literature values.[28] ^{1}H NMR (CD_3OD): δ = 7.59 (d, J = 8.0 Hz, 1H), 7.34 (d, J = 8.0 Hz, 1H), 7.13 (t, J = 8.0 Hz, 1H), 7.08–7.03 (m, 2H), 6.62 (d, J = 8.4 Hz, 2H), 6.46 (d, J = 8.4 Hz, 2H), 4.20–4.15 (brs?, 1H), 3.85 (dd, J = 8.4, 3.6 Hz, 1H), 3.04 (dd, J = 14.8, 4.4 Hz, 1H; AB), 2.78 (dd, J = 14.8, 4.4 Hz, 1H; AB), 2.56 (dd, J = 13.6, 3.6 Hz, 1H; AB), 1.45 ppm (dd, J = 13.6, 8.4 Hz, 1H; AB); ^{13}C NMR (CD_3OD): δ = 169.66, 169.32, 157.54, 138.02, 131.97, 128.90, 127.70, 125.80, 122.66, 120.22, 119.95, 116.16, 112.49, 109.62, 57.87, 57.06, 40.57, 31.21 ppm; HRMS: *m/z* calcd for $C_{20}H_{19}O_3N_3+H^+$: 350.1505 $[M+H]^+$; found: 350.1498.

Dipeptide Fmoc-D-Trp(Boc)-L-His(Tr)methyl ester (18): HATU (0.216 g, 0.57 mmol, 1.0 equiv) was added to a solution of Fmoc-D-Trp(Boc)COOH (0.300 g, 0.57 mmol, 1.0 equiv) in freshly distilled CH_2Cl_2 (3 mL). After 15–30 min, a solution of L-His(Tr)OMe (0.255 g, 0.57 mmol, 1.0 equiv) and NEt_3 (0.16 mL, 2.0 equiv) in freshly distilled CH_2Cl_2 (3 mL) was added dropwise. The reaction mixture was stirred overnight. After removal of the solvent, the mixture was purified on silica (MeOH in CH_2Cl_2, 1%) to afford the expected compound as an amorphous white solid (0.51 g, 99%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.10–8.05 (brs, 1H), 8.02–7.90 (brs, 1H; NH), 7.72 (m, 2H), 7.66–7.62 (brd, 1H), 7.56–7.48 (m, 3H), 7.40–7.16 (m, 16H), 7.08–7.04 (m, 6H), 6.53 (s, 1H), 5.80–5.70 (brs, 1H; NH), 4.72–4.66 (brs, 1H), 4.60–4.52 (brs, 1H), 4.32–4.21 (m, 2H), 4.18–4.11 (brs, 1H), 3.55 (s, 3H), 3.21–3.18 (m, 2H; AB), 3.10–2.95 (brs, 1H; AB), 2.79–2.75 (brs, 1H; AB), 1.58 ppm (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.11–170.75, 156.03, 149.57, 143.87, 143.76, 141.79, 141.22, 129.69, 128.26, 128.19, 127.72, 127.11, 125.23, 124.60,

124.47, 122.65, 119.95, 119.71, 119.06, 115.40, 115.26, 83.46, 67.28, 55.22, 52.63, 52.27, 47.05, 28.76–28.41, 28.18 ppm.

***Cyclo*-D-Trp(Boc)-L-His(Tr) (20)**: Dry piperidine (3 mL) was added to a solution of compound **18** (0.51 g, 0.56 mmol) in dry DMF (3 mL). The reaction mixture was stirred for 2 h, then quenched with water, and extracted with EtOAc. The organic layers were combined, washed with brine, and dried over $MgSO_4$. After removal of the solvent, the crude product was purified on silica (MeOH in CH_2Cl_2, 5%) to afford the expected compound as an amorphous white solid (0.23 g, 62%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.19–8.11 (br s, 1H), 7.57 (s, 1H; NH), 7.52–7.48 (m, 2H), 7.43–7.22 (m, 12H), 7.19 (t, J = 7.6 Hz, 1H), 7.11–7.06 (m, 6H), 6.61 (s, 1H), 6.10 (s, 1H; NH), 4.17–4.02 (m, 2H), 3.49 (dd, J = 2.8, 14.0 Hz, 1H; AB), 3.14 (dd, J = 3.2, 15.2 Hz, 1H; AB), 3.97 (dd, J = 2.8, 14.0 Hz, 1H; AB), 2.86 (dd, J = 3.2, 15.2 Hz, 1H; AB), 1.66 ppm (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 167.67, 167.11, 142.25, 138.85, 136.17, 129.78, 129.58, 128.28, 125.05, 122.99, 119.84, 119.01, 115.56, 114.52, 84.08, 55.12, 54.82, 31.40, 30.34, 28.30 ppm; LC-MS: m/z: 666.00 $[M+H]^+$.

D,L-HTD (3): The same procedure as described for L,L-HTD (**1**), followed by purification by HPLC, yielded the title compound as a white solid (0.10 g, 96%). Spectroscopic data were in accordance with known literature values.[24] $[\alpha]_D^{20} = -17$ (c = 4.84, H_2O); ^{1}H NMR (400 MHz, CD_3OD): δ = 7.57 (d, J = 8.0 Hz, 1H), 7.51 (s, 1H), 7.32 (d, J = 8.0 Hz, 1H), 7.11–7.05 (m, 2H), 7.01 (t, J = 8.0 Hz, 1H), 6.65 (s, 1H), 3.95 (t, J = 4.0 Hz, 1H), 3.40–3.35 (m, 1H; AB), 3.15–3.08 (m, 2H; AB), 2.98–2.68 ppm (m, 2H; AB); ^{13}C NMR (100 MHz, CD_3OD): δ = 170.89, 169.86, 137.95, 136.25, 128.78, 125.93, 122.56, 120.13, 119.72, 112.24, 109.14, 57.20, 55.23, 30.80 ppm; LC-MS: m/z: 324.07 $[M+H]^+$; HRMS: m/z calcd for $C_{17}H_{17}N_5O_2+H^+$: 324.1461 $[M+H]^+$; found: 324.1449.

Dipeptide Fmoc-D-Trp(Boc)-D-His(Tr) methyl ester (33): The same procedure as described above for compound **20** afforded the title compound as an amorphous white solid (0.40 g, 99%); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.10–8.05 (m, 1H), 7.74–7.12 (m, 23H), 7.08–7.04 (m, 6H), 6.59 (s, 1H), 6.05–5.97 (br s, 1H; NH), 4.80–4.71 (m, 1H), 4.64–4.55 (m, 1H), 4.29–4.04 (m, 3H; Fmoc*CH*–CH_2), 3.55 (s, 3H), 3.28–3.15 (m, 2H; AB), 3.05–3.02 (m, 2H; AB), 1.61 ppm (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.10–170.63, 156.09, 149.56, 143.87, 143.76, 141.79, 141.22, 129.69, 128.26, 128.19, 127.72, 127.11, 125.23, 124.60, 124.47, 122.65, 119.95, 119.71, 119.06, 115.40, 115.26, 83.51, 67.24, 55.22, 52.63, 52.27, 47.05, 28.76–28.41, 28.18 ppm.

***Cyclo*-D-Trp(Boc)-D-His(Tr) (34)**: Applying the procedure as described above for compound **22** afforded the title compound as an amorphous white solid (0.14 g, 57%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.11 (d, J = 8.0 Hz, 1H), 7.55–7.46 (m, 3H), 7.40–7.30 (m, 10H), 7.23 (t, J = 8.0 Hz, 1H), 7.16–7.08 (m, 7H), 6.48 (s, 1H), 5.95 (s, 1H; NH), 4.32–4.29 (m, 1H), 4.23–4.20 (m, 1H), 3.59–3.52 (m, 1H; AB), 3.22–3.15 (m, 1H; AB), 3.02–2.96 (m, 1H; AB), 2.50–2.40 (m, 1H; AB), 1.66 ppm (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 167.80, 166.99, 142.22, 138.95, 136.17, 129.83, 129.58, 128.28, 125.07, 123.03, 119.76, 119.01, 115.58, 114.59, 84.08, 55.05, 54.15, 31.03, 29.47, 28.31 ppm.

D,D-HTD (4): Applying the procedure as described for L,L-HTD (**1**), followed by HPLC purification, yielded the title compound as a white solid (0.14 g, 31%). Spectroscopic data were in accordance with known literature values.[24] $[\alpha]_D^{20} = +35$ (c = 2.56, H_2O); ^{1}H NMR (400 MHz, CD_3OD): δ = 7.63 (d, J = 7.6 Hz, 1H), 7.48 (s, 1H), 7.37 (d, J = 8.0 Hz, 1H), 7.17–7.04 (m, 3H), 5.79 (s, 1H), 4.26–4.20 (m, 1H), 3.92–3.86 (m, 1H), 3.34–3.22 (m, 1H; AB), 3.18–3.09 (m, 1H; AB), 2.48–2.41 (m, 1H; AB), 1.12–1.06 ppm (m, 1H; AB); ^{13}C NMR (100 MHz, CD_3OD): δ = 170.89, 169.86, 137.95, 136.53, 129.21, 126.15, 122.66, 120.28, 120.21, 112.51, 109.54, 57.28, 55.95, 30.80 ppm; LC-MS: m/z: 324.07 $[M+H]^+$; HRMS: m/z calcd for $C_{17}H_{17}N_5O_2+H^+$: 324.1461 $[M+H]^+$; found: 324.1449.

Dipeptide Fmoc-L-benzothiophenylalanine-L-His(Tr) methyl ester (35): Applying the procedure as described above for compound **20** afforded the title compound as an amorphous white solid (0.30 g, 65%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.90 (s, 1H), 7.82–7.66 (m, 6H), 7.49–7.18 (m, 20H), 7.11–7.02 (m, 6H), 6.78 (s, 1H), 5.84 (d, J = 6.4 Hz, 1H; NH), 4.78–4.70 (m, 1H), 4.59–4.51 (m, 1H), 4.24–4.02 (m, 3H; Fmoc*CH*–CH_2), 3.55 (s, 3H), 3.39–3.31 (m, 1H; AB), 3.27–3.05 ppm (m, 3H; AB); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.59, 169.88, 165.68, 156.44, 143.64, 143.60, 141.13, 140.33, 139.98, 138.65, 136.08, 131.17, 130.50, 128.57, 128.94, 128.71, 128.64, 127.97, 127.85, 127.73, 127.14, 126.92, 125.27, 125.19, 124.68, 124.40, 124.27, 122.78, 121.56, 119.89, 78.33, 67.48, 55.07, 52.70, 52.04, 46.77, 30.51, 27.19 ppm.

***Cyclo*-L-benzothiophenylalanine-L-His(Tr) (36)**: Applying the procedure as described above for compound **22** afforded the title compound as an amorphous white solid (0.19 g, 89%); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.80 (d, J = 8.0 Hz, 1H), 7.67 (d, J = 8.0 Hz, 1H), 7.60 (s, 1H; NH), 7.38–7.01 (m, 19H), 6.16 (s, 1H), 4.38–4.31 (m, 1H), 4.29–4.10 (m, 1H), 3.51–3.41 (m, 1H; AB), 3.37–3.25 (m, 1H; AB), 2.95–2.88 (m, 1H; AB), 1.82–1.71 ppm (m, 1H; AB); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 168.11, 166.75, 142.12, 140.18, 138.78, 138.63, 135.81, 130.34, 129.69, 128.11, 125.71, 124.42, 124.35, 122.63, 122.06, 119.95, 75.39, 54.71–54.64, 32.52, 31.80 ppm.

***Cyclo*-L-benzothiophenylalanine-L-His (7)**: Applying the procedure as described for L,L-HTD (**1**), followed by HPLC purification, yielded the title compound as a white solid (0.066 g, 62%). $[\alpha]_D^{20} = -33$ (c = 11.8, H_2O); ^{1}H NMR (400 MHz, D_2O): δ = 8.30 (d, J = 1.2 Hz, 1H), 8.00–7.94 (m, 1H), 7.84–7.79 (m, 1H), 7.52–7.44 (m, 2H), 7.37 (s, 1H), 6.13 (d, J = 1.2 Hz, 1H), 4.52 (dd, J = 1.2, 4.8 Hz, 1H), 4.07 (dd, J = 1.2, 4.8 Hz, 1H), 3.52 (dd, J = 4.8, 15.2 Hz, 1H; AB), 3.26 (dd, J = 4.8, 15.2 Hz, 1H; AB), 2.39 (dd, J = 4.8, 15.2 Hz, 1H; AB), 1.67 ppm (dd, J = 8.0, 15.2 Hz, 1H; AB); ^{13}C NMR (100 MHz, D_2O): δ = 169.11, 167.46, 139.84, 138.75, 133.40, 129.63, 127.10, 126.64, 124.76–124.67, 123.18, 122.18, 177.33, 55.13, 53.16, 31.09, 28.28 ppm; HRMS: m/z calcd for $C_{17}H_{16}N_4O_2S+H^+$: 341.1072 $[M+H]^+$; found: 341.1076.

Dipeptide Fmoc-L-naphthylalanine-L-His(Tr) methyl ester (37): Applying the procedure as described above for compound **20** afforded the title compound as an amorphous white solid (0.17 g, 58%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.81–7.68 (m, 7H), 7.60–7.49 (br s, 1H; NH), 7.47–7.18 (m, 23H), 7.09–7.03 (m, 6H), 6.61 (s, 1H), 5.61–5.59 (br s, 1H; NH), 4.82–4.76 (m, 1H), 4.67–4.59 (m, 1H), 4.31–4.24 (m, 1H; Fmoc*CH*–CH_2), 4.19–4.07 (m, 2H; FmocCH–CH_2), 3.52 (s, 3H), 3.49–3.42 (m, 1H; AB), 3.21–3.17 (m, 1H; AB), 3.08–3.01 ppm (m, 2H; AB); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.10, 170.59, 156.25, 143.85–143.81, 141.31–141.28, 133.86, 133.62, 132.59, 129.76, 128.85–127.02, 126.29–125.23, 120.63, 120.00, 67.47, 56.15, 52.56, 52.41, 47.06, 38.47, 28.05 ppm; LC-MS: m/z: 831.13 $[M+H]^+$, 1661.80 $[2M]^+$.

***Cyclo*-L-naphthylalanine-L-His(Tr) (38)**: Applying the procedure as described above for compound **22** afforded the title compound as an amorphous white solid (0.07 g, 58%). ^{1}H NMR (400 MHz, CD_3OD): δ = 7.62–7.50 (m, 3H), 7.42–7.32 (m, 9H), 7.28–7.21 (m, 3H), 7.19–7.14 (m, 1H), 7.11–7.03 (m, 6H), 6.96–6.91 (m, 1H), 5.59 (s, 1H), 4.39–4.33 (br s, 1H), 3.99–3.95 (m, 1H), 3.47–3.30 (m, 1H; AB), 3.12–3.04 (m, 1H; AB), 2.16 (dd, J = 4.4, 14.0 Hz, 1H; AB), 0.64–

0.56 ppm (m, 1H; AB); LC-MS: *m/z*: $[M+H]^+ = 577.00$; $[2M]^+ = 1152.93$.

***Cyclo*-L-naphthylalanine-L-His (8)**: Applying the procedure as described for L,L-HTD (**1**), followed by HPLC purification, yielded the title compound as a white solid (0.019 g, 49%)); $[\alpha]_D^{20} = -39.7$ ($c = 2.82$, H_2O); ^1H NMR (400 MHz, D_2O): $\delta = 7.95–7.85$ (m, 3H), 7.74 (d, $J = 0.8$ Hz, 1H), 7.64–7.53 (m, 3H), 7.28 (dd, $J = 2.0$, 8.4 Hz, 1H), 6.38 (s, 1H), 4.55 (dd, $J = 3.6$, 4.8 Hz, 1H), 4.13 (dd, $J = 4.8$, 6.8 Hz, 1H), 3.37 (dd, $J = 3.6$, 14.0 Hz, 1H; AB), 3.10 (dd, $J = 4.8$, 14.0 Hz, 1H; AB), 2.31 (dd, $J = 4.8$, 15.6 Hz, 1H; AB), 1.81 ppm (dd, $J = 6.8$, 15.6 Hz, 1H; AB); ^{13}C NMR (100 MHz, D_2O): $\delta = 169.06$, 167.59, 133.02, 132.98, 132.41, 132.23, 129.07, 128.76, 128.06, 127.72, 127.70, 126.78, 126.58, 126.50, 117.29, 55.66, 53.10, 37.98, 27.76 ppm; HRMS: *m/z* calcd for $C_{19}H_{18}N_4O_2 + H^+$: 335.1508 $[M+H]^+$; found: 335.1511.

Acknowledgements

This work was supported by the Perspective Genbiotics program subsidized by STW (Stichting Technische Wetenschappen).

Keywords: alkaloids · antibiotics · diketopiperazines · mutasynthesis · *Penicillium* · roquefortines

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Received: December 1, 2014
Published online on March 12, 2015