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Synthesis of a Cyanopeptide-Analogue with Trypsin Activating Properties

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Abstract—An efficient synthesis of a peptidic analogue of cyanobacterial metabolites with proposed serine protease inhibitory activity has been developed. Surprisingly, one trypsin activating compound was obtained.

Cyanobacteria are well-known sources of interesting metabolites, many of which possess significant biological activity.[1] Thus, cyanobacteria have been subjected to systematical screenings. Cyanopeptides show in addition to hepato- and neurotoxic properties a broad spectrum of biological activities, including antitumor,[2] immunosuppressive,[3] and antimicrobial[4] effects as well as angiotensin-converting enzyme inhibitory action[5] and cardioactive effects.[6]

Besides the hepatotoxic cyclic peptides (microcystines), cyanobacteria of the genera *Microcystis*, *Oscillatoria*, and *Anabaena* usually produce non-toxic peptides that inhibit serine proteases, which play a central role in the human organism.[7–10] Failures of one or more of these enzymes may cause a state of imbalance between protease and antiprotease (e.g. endogenous protease inhibitors) and can lead to an excess of proteolytic activity and/or to the development of diseases such as pancreatitis.[11]

Because of their biological potency, the linear cyanopeptides aeruginosin 98-A, aeruginosin 98-B (**1**, **2**; from *Microcystis aeruginosa*)[12] and aeruginosin 102-A (**3**, *Microcystis viridis*)[7] could be considered as new lead structures for the design and synthesis of new selective serine protease inhibitors. Each of these compounds inhibits trypsin, thrombin, and plasmin. Compound **3** is the strongest inhibitor of all (IC_{50}: 0.2/0.04/0.3 μg/mL), while **1** and **2** are equipotent (IC_{50}: 0.6 and 0.6/7.0 and 10.0/6.0 and 7.0 μg/mL).

Reversed-phase HPLC and Chiral GC analysis have confirmed that **1** and **2** contain no standard L-amino acids.[12] Isoleucine shows D-allo configuration; if the residue was a L- or a L-allo-isoleucine, the side-chain would produce steric conflicts with the bulky 2-carboxy-6-hydroxy-octahydroindole (Choi) sulfate. Agmatine constitutes the basic side-chain and seems to be essential for inhibiting the above mentioned serine proteases. The relative stereochemistry of the Choi-sulfate was deduced by NMR studies; the absolute configuration of **2** was determined by means of X-ray crystallographic methods of the cocrystallized **2**-trypsin-complex (Brookhaven Protein Data Bank, code: 1aq7).[13] The X-ray data provide information about atypical trypsin-inhibitor interactions, which are caused by the non-peptidic partial structure of **2** and mainly based on usual hydrogen bonds and van der Waals interactions. Furthermore, there is evidence for other interactions such as the sulfate moiety protruding into the solvent or the strong, water-mediated interaction between the hydroxy group of the 4-hydroxyphenyllactic acid (Hpla) and the amino acids Cys220 and Ser146 of trypsin.

The conspicuous lack of any interactions of **2** with trypsin's catalytic triade, Ser195, His57, and Asp102, is unique. The Ser195 residue does not show close contacts to any atoms of **2**, this discovery may mean that a new mode of inhibition differing from the standard mechanisms of serine protease inhibitors is possible.[13]

Both the diversity of the above mentioned interaction of **2** with trypsin and the discussion of a new inhibiting mechanism of serine proteases justify additional investigations of structure activity relationships between

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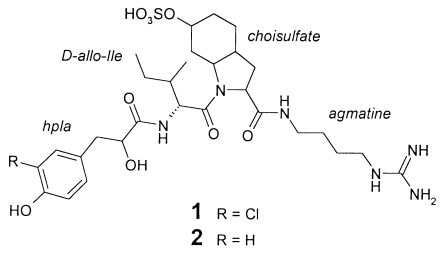
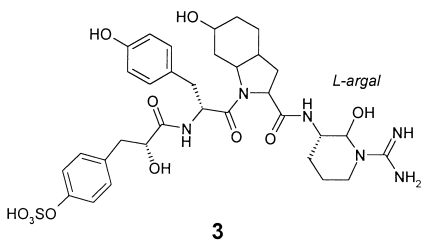




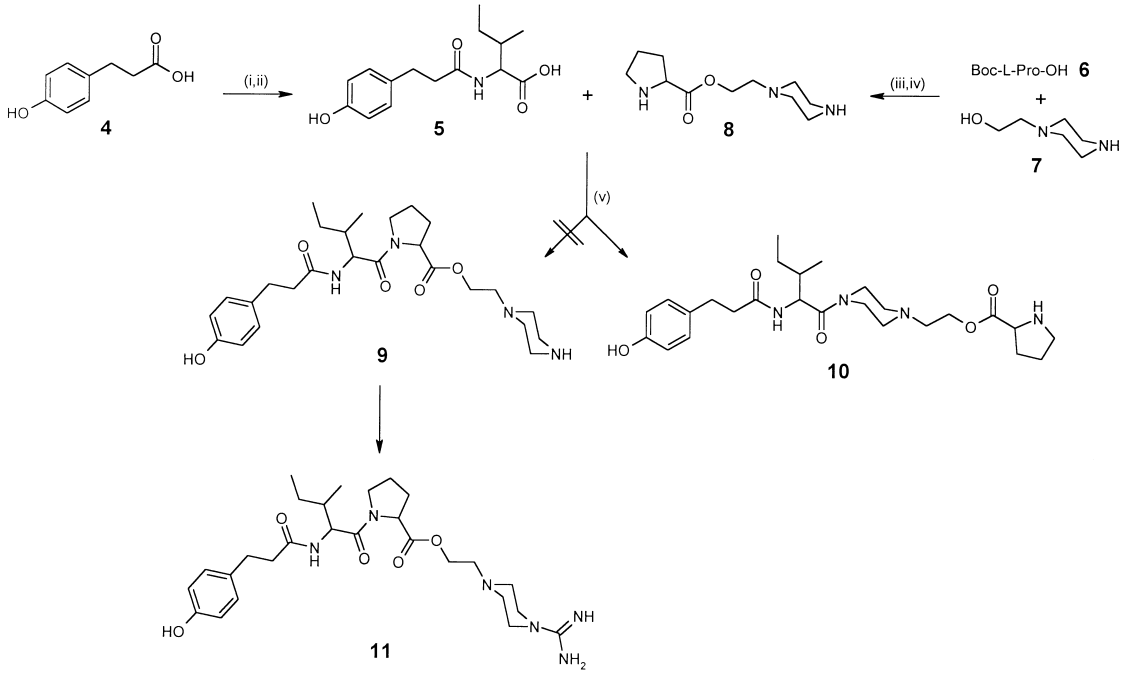
serine proteases such as trypsin and its effectors. In this paper, we describe the synthesis and inhibition tests of the trypsin effector **10** by taking **2** into consideration as a lead structure (Scheme 1).

In order to simplify the stereochemical obstacles in **2** on the route to useful synthetic analogues, e.g., the target molecules **9** and **11**, we substituted the chiral Hpla unit in **2** by 3-(4-hydroxyphenyl)-propionic acid (**4**) and decreased the sterically hindered Choi moiety to L-proline. Through these modifications, we circumvent the necessity of using the expensive D-allo-isoleucine and were able to applicate L-isoleucine. To provide a basic C-terminal side-chain, which is essential for the inhibition of trypsin, agmatine in **2** was exchanged for 2-(1-piperazino)ethanol, since syntheses of the extraordinarily expensive agmatine only succeeded in very low yields. If this side-chain turns out to be not basic enough to inhibit trypsin effectively, it will have to be transformed into the appropriate guanyl derivative **11**.

Thus, the desired building blocks **5** and **8** were synthesized by using standard methods in peptide synthesis (Scheme 1). 3-(4-Hydroxyphenyl)-propionic acid (**4**) was transformed into the appropriate active ester by esterification with *N*-hydroxysuccinimide and then subjected to the completely unprotected L-isoleucine to give **5**. Compound **8** was obtained by DMAP/DCC-catalyzed esterification of Boc-L-proline (**6**) with 2-(1-piperazino)-ethanol (**7**) followed by the Boc-cleavage by the means of TFA. DCC-catalyzed coupling of **5** with **8** exclusively lead to the formation of **10**, whose structure has been proved by NMR and MS studies.[14]

Surprisingly, **10** turned out to be a trypsin activator instead of behaving as an inhibitor. The inhibition tests were performed by using *N*-α-benzoyl-DL-arginyl-4-nitroanilide (BAPNA) as the substrate of trypsin. BAPNA was cleaved into *N*-α-benzoyl-DL-arginine and 4-nitroaniline, whose absorption at 405 nm was measured as an indicator of enzyme activity. Compound **10**, at a concentration between 20 and 40 mM, increases the activity of trypsin by 70%. Whether this effect depends on the concentration of BAPNA (1 mg/mL) and/or is specific for BAPNA has to be clarified by further tests.

In conclusion, we have developed a synthesis of a new serine protease activator in high yields under moderate



Scheme 1. (i) HOSu/DCC/EA, 78%; (ii) $NaHCO_3$/L-Ile-OH/EtOH, 76%; (iii) DMAP/DCC/DCM, 70%; (iv) TFA/$NaHCO_3$, quant.; (v) DCC/EA, 56% Abbreviations: DCC: *N,N*-dicyclohexylcarbodiimide; DCM: dichloromethane; DMAP: *N,N*-dimethyl-amino-pyridine; EA: ethyl acetate; HOSu: *N*-hydroxysuccinimide; TFA: trifluoroacetic acid.

conditions. Further studies of the structure activity relationship and the mechanism of the activation have to follow.

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14. **5**: ^{1}H NMR (200 MHz, DMSO-d_6): δ (ppm) = 0.80 (t, 3 H, Ile-CH_2-CH_3); 0.83 (t, 3 H, Ile-CH–CH_3); 1.14 (m, 1 H, Ile-CH_2); 1.31 (m, 1 H, Ile-CH_2); 1.70 (m, 1 H, Ile-CH); 2.34 (t, 2 H, PhCH_2–CH_2–COOR); 2.68 (t, 2 H, PhCH_2–CH_2–COOR); 4.16 (t, 1 H, Ile-H_α); 6.64 (d, 8.0 Hz, 2 H, $H_{arom.}$); 6.98 (d, 8.0 Hz, 2 H, $H_{arom.}$); 7.97 (d, 8.0 Hz, 1 H, Ile-NH); 9.13 (s, 1 H, OH). ^{13}C NMR (70 MHz, DMSO-d_6): δ (ppm) = 11.1 (CH_3CH_2, Ile); 16.3 (CH_3CH-, Ile); 24.6 (CH_2, Ile); 30.3 (Ph–CH_2–CH_2-R); 36.2 (Ph–CH_2–CH_2-R); 36.9 (β-CH, Ile); 56.0 (α-CH, Ile); 114.9 ($CH_{arom.}$); 129.0 ($CH_{arom.}$); 131.2 (C-$1_{arom.}$); 155.3 (C-$4_{arom.}$); 171.7 (CONHR); 173.1 (COOH) $[\alpha]_D^{20}$ −11.5° (MeOH; $c = 2$); mp: 122–126 °C (H_2O). Compound **8** was generated in situ from the appropriate *N*-Boc-proline derivative and was subjected to **5** without further purification. Compound **10**: NMR studies (300 MHz, MeOH-d_4) could not distinguish between the structures of **9** and **10**. With the help of MS we were able to assign the fragmentation scheme to the structure of **10**. MS (EI, 70 eV): m/z (intensity %) = 488 (M^+, 18), 360 (15), 262 (29), 234 (30), 128 (8), 127 (5), 107 (44), 98 (15), 70 (basis, 100).