Serine Protease Inhibitors

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Chemistry and Biology of the Aeruginosin Family of Serine Protease Inhibitors

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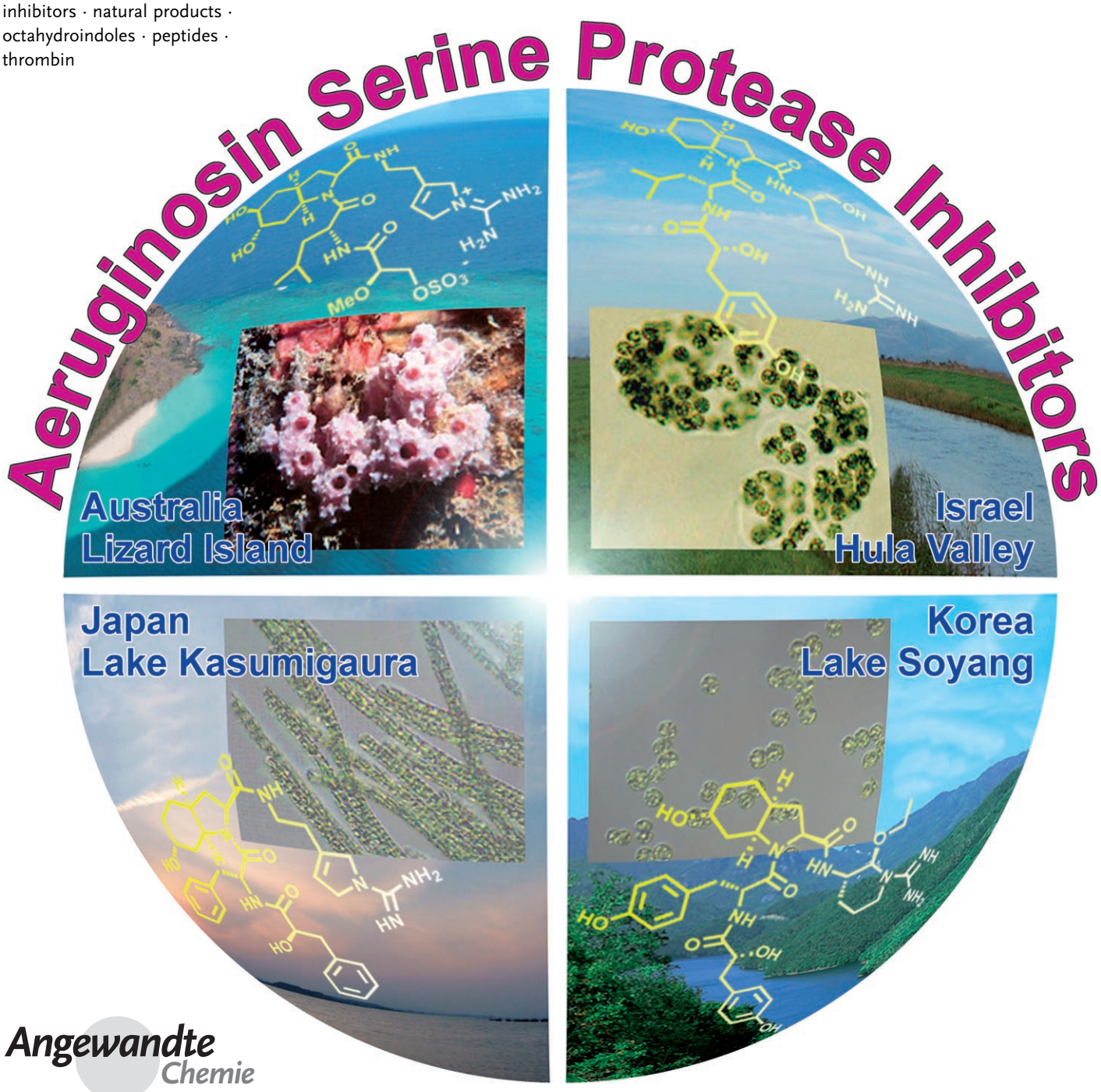

The aeruginosins have been isolated from marine sponges and cyanobacterial waterblooms, sources that are phylogenetically distinct and the bodies of water are geographically well-separated. The aeruginosins comprise a central hydroxy- (or dihydroxy-) octahydroindole carboxamide core unit, onto which are appended unusual amino acids on the carboxy and amino termini as part of the linear peptide array. Potent inhibitory activity of serine proteases in vitro is exhibited by some of the aeruginosins as a result of the presence and proper deployment of three important pharmacophoric subunits: a P1 arginine mimetic, and two hydrophobic residues with interaction sites designated as P2 and P3. In this article, we provide the first comprehensive review on the chemistry and biology of the aeruginosins, with an emphasis on their sources, structural revisions, and total syntheses.

From the Contents

1. Introduction

Cyanobacterial waterblooms have long been a source of biologically interesting secondary metabolites.[1–4] They have been named as such owing to the cyan color of the algae from which they are derived. Hepatotoxic cyclic peptides such as the microcystins[5–7] and nodularins[8,9] are largely responsible for the health risks associated with these algal blooms.[10–12] Reports of contaminated water supplies and outbreaks of livestock poisoning have motivated researchers to more closely examine the compounds produced by freshwater blue-green algae. Among the more interesting findings emanating from their studies has been the identification of a new class of linear peptides called aeruginosins, which exhibit inhibitory activity against serine proteases. In the course of further biological screening of metabolites produced by *Microcystis aeruginosa*, Murakami and co-workers[13] reported the isolation of aeruginosin 298A in 1994. This marked the introduction of a new class of peptidic serine protease inhibitors to the chemistry community. Over the past decade, 20 more compounds structurally and pharmacologically related to aeruginosin 298A have been isolated. Some of these have been gathered from geographically distinct locations and from sources having an unclear relationship to the *Microcystis* waterbloom.

The aeruginosins exhibit varying degrees of inhibitory activity against serine proteases, and their activity profile can be explained by a high degree of pharmacophoric and structural homology within the family. Nearly all of the aeruginosins are composed of four subunits: an N-terminal hydroxy or acidic group, a bulky hydrophobic amino acid, a 2-carboxyperhydroindole core, and a C-terminal guanidine-containing group (Figure 1). This array of structural and functional features is responsible for their affinity to the catalytic binding pocket of trypsin, thrombin, and other serine proteases involved in the blood coagulation cascade.

The aeruginosins have also garnered considerable attention from synthetic organic chemists as a result of their structural novelty and biological activity. Although these compounds are composed of similar subunits, isolation of new members has often been attended by the discovery of previously unknown amino acids and arginine-mimetic subunits as constituents. Derivatives of the 2-carboxyperhydroindole core structure have received the most attention with respect to conceptually diverse synthetic approaches. To date, the total syntheses of seven aeruginosins have been completed, four of these involving revisions to the originally proposed structures.

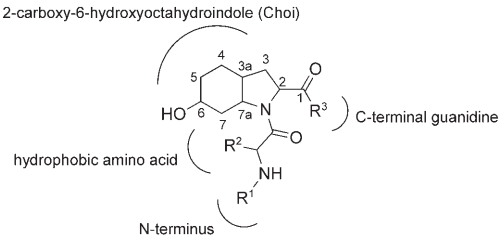

Figure 1. Generalized structure of the aeruginosins.

The aim of this article is to present an overview of the chemistry and biology of the aeruginosins. A summary of their isolation and characterization is followed by a discussion of biological activity and synthetic efforts. Particular attention

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is paid to the development of new methodologies applied to the total synthesis of selected aeruginosins.

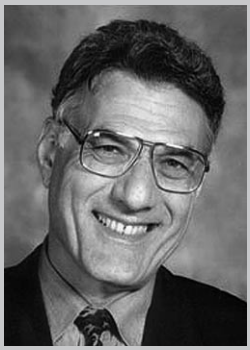
2. Isolation and Characterization

Toxic and nontoxic strains of blue-green algae, particularly those produced by the cyanobacterium of the genus *Microcystis*, have been a prodigious source of structurally unique and biologically active peptides. Efforts to identify the causes of toxicity associated with these algal blooms have resulted in the isolation of several nontoxic peptides exhibiting potent inhibitory activity against serine proteases. Among them were the first known members of the aeruginosin family of natural products.

Initially, the aeruginosins were classified as natural products produced by the cyanobacterium *Microcystis aeruginosa* that incorporated a new azabicyclic amino acid, 2-carboxy-6-hydroxyoctahydroindole (Choi), whose general structure is depicted in Figure 1. In the years following the isolation of the thrombin and trypsin inhibitor aeruginosin 298A (**1**),[13] linear peptides containing the core bicyclic subunit and having similar biological activities and structural features were isolated from the *Oscillatoria* genus of freshwater cyanobacteria as well as from marine sponges of the family *Dysideidae*. Despite their diverse origins, the 21 compounds herein considered to be members of the aeruginosin family all contain the distinctive *cis*-fused 2-carboxyperhydroindole core structure, and with a few exceptions they exhibit in vitro inhibitory activity against serine proteases. Additional cyanopeptides, structurally belonging to the aeruginosin family, such as aeruginosins 602, 670, and 678, have also been identified from *Microcystis* colonies.[14–17] However, to the best of our knowledge these peptides have not been fully characterized nor tested biologically against serine proteases, and they are therefore not included here.

Karolina Ersmark received her MSc in pharmacy from the Faculty of Pharmacy, Uppsala University (Sweden) in 1999. In 2000, she joined Professor Anders Hallberg at the Department of Medicinal Chemistry, Uppsala University, and received her PhD in 2005, with a thesis entitled "Design and Synthesis of Malarial Aspartic Protease Inhibitors". She then joined Professor Stephen Hanessian at Université de Montréal (Canada) and started to work in the field of the aeruginosins. She is currently a research scientiest at Medivir (Sweden).

Juan R. Del Valle was born in Milwaukee (USA) in 1977. He received his PhD in 2004 from the University of California, San Diego, under Professor Murray Goodman. From 2004 to 2006 he was a postdoctoral research associate in the laboratory of Professor Stephen Hanessian, where he conducted research on the synthesis of peptide and alkaloid natural products. His research interests focus on the synthesis of biologically active peptidomimetics and natural products, and the development of new synthetic methods. He is currently Assistant Professor of Organic Chemistry at New Mexico State University.



Stephen Hanessian holds the Achaogen Research Chair in the Department of Chemistry at the Université de Montréal. Since 2000, he has also been Adjunct Professor of Chemistry and Pharmaceutical Sciences at the University of California, Irvine, where he will direct a new interdisciplinary graduate program in medicinal chemistry and pharmacology starting in 2008. Professor Hanessian's research interests include natural products synthesis, various aspects of medicinal chemistry, organic synthetic methodology, catalysis and asymmetric processes, molecular recognition, carbohydrates and peptides, and computer-assisted synthesis.

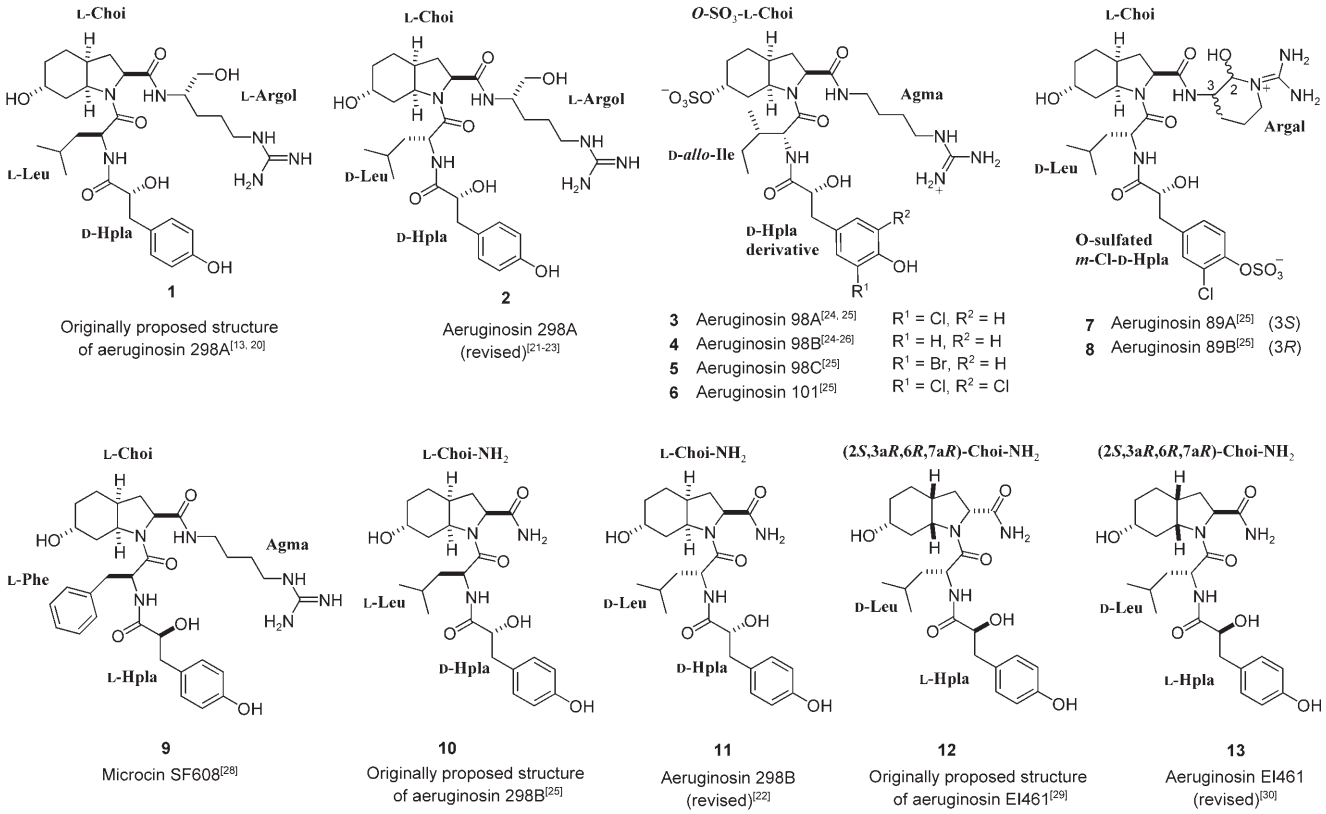
2.1. *Aeruginosins from* Microcystis aeruginosa

In the early 1990s Murakami and co-workers[18,19] reported the isolation and characterization of the novel protease inhibitory peptides microginin and micropeptins A and B from *Microcystis aeruginosa*. This was soon followed by the isolation of aeruginosin 298A (**2**) from the same cyanobacterial source.[13] The structure of aeruginosin 298A (**1**) was initially elucidated through 2D NMR studies, and the peptide was found to contain four subunits: hydroxyphenyllactic acid (Hpla), leucine, a Choi core, and a reduced arginine (Argol). In their initial report, Murakami and co-workers[13] assigned an L configuration to the leucine amide subunit in aeruginosin 298A based on degradation and derivatization of the acid hydrolyzate.

In 1998 Tulinsky and co-workers[20] published an X-ray crystallographic structure of a ternary complex of **1** bound to a hirugen–thrombin complex at 2.1-Å resolution, thus providing an absolute stereochemical configuration. The Hpla subunit was proposed to display a D configuration and the leucine an L configuration. The stereogenic centers in the substituted 2-carboxy-6-octahydroindole (Choi)[13] amino acid were designated as 2*S*,3a*S*,6*R*,7a*S*, and the absolute stereochemistry as L-Choi, while the Argol subunit was found to also have an L configuration.

The crystal structure of the ternary complex revealed some unexpected interactions between **1** and the binding pocket of thrombin. L-Leu was found to occupy the hydrophobic D-enantiomorphic S3 subsite, despite its proposed L-stereochemical configuration. In addition, the Hpla residue was found to interact with the S_3 subsite, which was expected to accommodate the L-leucine residue. These key observations cast some doubt on the stereochemical assignments initially made for aeruginosin 298A. The issue would not be resolved until the proposed structure (**1**) was revised through total synthesis by the groups of Bonjoch[21,22] and Wipf[23] independently in 2000 (see revised structure **2**).

In the following years, Murakami and co-workers[24,25] reported the isolation and characterization of aeruginos-



sins 98A (also known as taihunosin), 98B, 98C, and 101 (**3**–**6**) from *Microcystis aeruginosa*. The structures were elucidated on the basis of 2D NMR spectroscopy, and absolute configurations were determined by acid hydrolysis of the peptide followed by derivatization and HPLC analysis on a chiral support for which standard samples served as controls. The absolute configuration of the Choi subunit was determined by derivatization and NMR analysis. The absolute configuration of aeruginosin 98B was determined by an X-ray analysis of a ternary complex with hirugen and thrombin.[26] Each of the structures was found to contain a D-Hpla residue, which in the case of aeruginosins 98A, 98C, and 101 contains a *meta*-bromo or -chloro substituent on the aromatic ring. The presence of a D-*allo*-isoleucine residue (where *allo*Ile has the opposite side-chain chirality as Ile), an O-sulfated L-Choi core subunit, and a 4-amidinobutylamide (agmatine, Agma) chain is common to each of the four structures.

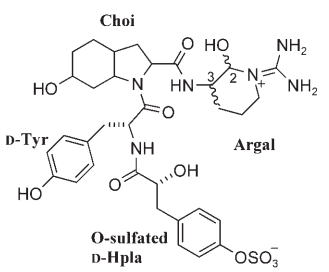
Aeruginosins 89A and 89B (**7**, **8**) were isolated in 1999 from a new strain of *Microcystis aeruginosa*, and their structures and absolute configurations were determined as described above.[25] These peptides were found to incorporate an unusual C-terminal argininal (Argal) residue in addition to a sulfated *m*-chloro-D-Hpla residue in their structures. The tautomeric equilibrium of the Argal moiety complicated the separation of these compounds as well as their stereochemical assignment. The peptide degradation products were therefore subjected to $NaClO_2$ oxidation to afford arginines, which were analyzed by HPLC as their Marfey derivatives[27] (precolumn derivatization with the chiral reagent 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide). The results suggested that aeruginosins 89A and 89B contain the L and D forms of argininal, respectively.

In the same year, while screening for protease inhibitors from a nontoxic strain of *Microcystis aeruginosa*, Carmeli and co-workers[28] discovered microcin SF608 (**9**). By combination of 2D NMR and HPLC analysis the structure of microcin SF608 was fully elucidated and found to closely resemble that of aeruginosin 298A. Microcin SF608 is made up of Agma, L-Choi, L-Phe, and L-Hpla subunits. Thus, the occurrence of an L-Phe residue in microcin SF608 appears to be an exception since all the amino acid residues in other aeruginosins have been revised to their D configurations.

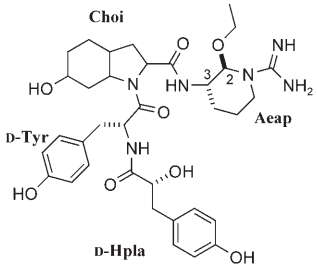
The two aeruginosins most recently isolated from *Microcystis aeruginosa* share the same Choi structure and lack the usual guanidine-containing C-terminal pharmacophores. Full characterization of aeruginosins 298B[25] (**11**) and EI461[29] (**13**) showed the peptides to be composed of L-Hpla, D-Leu, and a C-terminal Choi amide. Close inspection of NOE correlations from the Choi amide of aeruginosin EI461 revealed an axial hydrogen atom (H-6) at C6 and a *cis*-fused perhydroindole. The fact that H-6 showed an NOE correlation to the ring-junction hydrogens (H-3a and H-7a) suggested a Choi configuration unique among the aeruginosins. Comparisons with synthetic 6α and 6β isomers of Boc-L-Choi-OMe supported the stereochemical assignment 2*S*,3a*R*,6*R*,7a*R*.[30] Aeruginosin EI461 remains the only member of this family of natural products with an *anti* relationship between H-2 and H-3a/H-7a. The originally proposed structures of aeruginosins 298B (**10**) and EI461 (**12**) have both been revised through total synthesis.[22,30]

2.2. *Aeruginosins from* Microcystis viridis

Cultures of the freshwater cyanobacterium *Microcystis viridis* yielded three new members of the aeruginosin family. In 1996 aeruginosins 102A (**14**) and 102B (**15**) were isolated



14 Aeruginosin 102A[31] (3*S*)
15 Aeruginosin 102B[31] (3*R*)



16
Aeruginosin 103A[32]

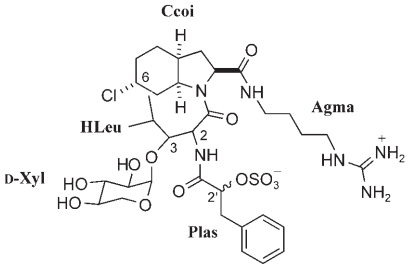
by ODS HPLC resolution of biologically active extracts.[31] As was the case with aeruginosins 89A and 89B, HPLC analysis showed a set of multiple peaks for each compound which persisted even when the compounds were separated and reanalyzed. This indicated a tautomeric equilibrium arising from the presence of an argininal subunit. Aeruginosins 102A and 102B were found to incorporate L- and D-Argal residues, respectively. In addition, both compounds harbor a D-Hpla sulfate, D-Tyr, and Choi subunits in their structures. Although the absolute configuration of the Choi in aeruginosin 102A and 102B has not been determined, NOE studies suggest a relative configuration matching that of the L-Choi units found in other aeruginosins.

Aeruginosin 103A (**16**) was isolated two years later and analyzed by NMR spectroscopy and peptide degradation.[32] Derivatization of the hydrolyzates revealed D-Hpla and D-Tyr subunits. Two-dimensional NMR spectroscopy showed the presence of a Choi residue whose relative stereochemistry matches that of the Choi subunits in aeruginosins 102A and 102B; however, the absolute stereochemistry of this subunit has not been established.

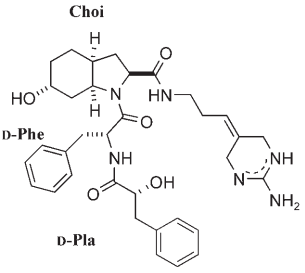
The defining feature of aeruginosin 103A is the presence of a cyclic ethyl hemiaminal found in the 1-amidino-2-ethoxy-3-aminopiperidine (Aeap) subunit. Interpretation of coupling constants and NOE correlations from the Aeap residue revealed a *syn* relationship between H-2 and H-4, and H-3 and H-5, all of which are axial hydrogens. To establish the absolute stereochemistry of Aeap, aeruginosin 103A was oxidized with CrO_3 in AcOH and the hydrolyzates analyzed by ODS HPLC. Identification of an L-Arg residue led to the assignment of a 1*R*,2*S* configuration for Aeap.

2.3. *Aeruginosins from* Oscillatoria *sp.*

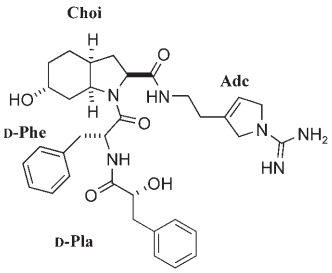
In 1997 Murakami and co-workers[33] isolated the first aeruginosins from beyond the genus *Microcystis*. The cyanobacterium *Oscillatoria agardhii* was collected from Lake Kasumigaura in Japan and mass-cultured to yield extracts with significant inhibitory activity against trypsin and thrombin. Purification by RP-HPLC afforded two new aeruginosins, 205A (**17**) and 205B (**18**). A characteristic isotopic and fragmentation pattern in the positive-ion FAB mass spectrum



Originally proposed structures of
17 Aeruginosin 205A[33] (2*R*,3*S*,2′*S*)
18 Aeruginosin 205B[33] (2*S*,3*R*,2′*R*)



19
Originally proposed structure of Oscillarin[41]



20
Oscillarin (revised)[42, 43]

indicated the presence of a chlorine atom as well as a sulfate group. Extensive 2D NMR experiments led to the identification of five subunits with the following proposed structures: phenyllactic acid-2-*O*-sulfate (Plas), 3-hydroxyleucine (Hleu), 2-carboxy-6-chlorooctahydroindole (Ccoi), Agma, and xylopyranose (Xyl).

Although the relative stereochemistry of the Ccoi residue was determined from the NMR coupling constants and by NOE analysis, its absolute configuration has not been established. Hydrolysis of the peptides and derivatization of the Plas residues as their menthyl esters showed an L- and D-Plas configuration for aeruginosins 205A and 205B, respectively. The Hleu residues were assigned as 2*R*,3*S* and 2*S*,3*R*, respectively, by Marfey analysis of the hydrolyzates. An assignment of D-xylose arose from GC analysis of the degradation fragment on a chiral column.

Aeruginosins 205A and 205B were the only known glycopeptidic aeruginosins at the time of their discovery, and they are among a very small group of sugar-containing natural products isolated from cyanobacteria.[1,2,34–38] The elucidation of their structures also resulted in the identification of two new structural units, namely Ccoi and Plas, and a third residue, Hleu, quite rare among natural products.

A controversy exists over the structures of aeruginosins 205A and 205B, which have been called into question by definitive synthesis of the Ccoi core.[39] Comparison of NMR data of synthetic Ccoi and that of the natural products show a

number of chemical-shift discrepancies, particularly in the vicinity of the chlorine atom. For example, in the ^{1}H NMR spectrum the H-6 methine proton of synthetic Ccoi shows a signal at $\delta = 4.60$, while in the spectrum of the natural product the resonance for this proton is recorded at $\delta = 3.83$. Since there are also significant differences in the ^{13}C NMR spectrum, it is likely that the chlorine atom of aeruginosins 205A and 205B is not on the 2-carboxyperhydroindole (Choi) core. In addition, the sulfate group, originally thought to be on the Plas residue, is now believed to be a part of the α-glycoside subunit.[40]

In the same year as the reported isolation of aeruginosins **17** and **18**, researchers at Boehringer Mannheim GmbH (now Roche Diagnostics) in Germany disclosed the isolation and characterization of oscillarin (**19**), also from algal cultures of *Oscillatoria agardhii* obtained from the Institute of Plant Physiology at the University of Göttingen, Germany, and originally isolated from Lake Kasumigaura in Japan.[41] The structure and absolute configuration were proposed on the basis of NMR data and a partially resolved cocrystal complex with trypsin. Oscillarin was proposed to comprise D-phenyllactic acid (D-Pla), D-Phe, L-Choi, and a cyclic guanidine-containing P1 subunit (see **19**). The structure was later revised to be **20** without changing the original data.[42]

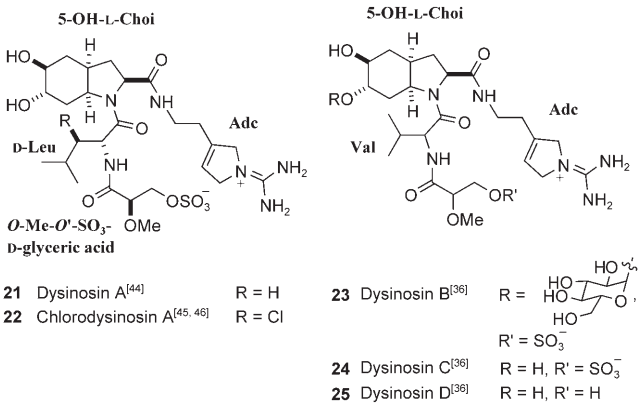
The revised structure of oscillarin (**20**), determined by a total synthesis and further confirmed by a high-resolution X-ray structure of a thrombin–oscillarin complex, revealed the presence of a 1-(*N*-amidino-Δ^3-pyrrolino)ethyl subunit (Adc) rather than the originally proposed cyclic guanidine.[43] This unique Adc heterocyclic motif is also present in the dysinosins[36,44] (discussed in the next section) and other linear peptides containing an azabicyclononane core subunit, such as suomilide,[37] and the recently isolated banyasides A and B.[38]

2.4. Aeruginosins from the Dysideidae Sponges

A new genus and species of sponge belonging to the family *Dysideidae* was discovered near Lizard Island in North Queensland, Australia. The extracts of this sponge yielded a peptidic thrombin inhibitor with significant structural similarities to the aeruginosins. Dysinosin A (**21**), whose isolation and characterization were reported by Quinn and co-workers in 2002,[44] is a functionally novel aeruginosin originating from a distinct geographic region.

The structural elucidation of dysinosin A was achieved through a combination of NMR and degradation studies, while stereochemical assignments were confirmed by the X-ray structure of a ternary complex of dysinosin A, thrombin, and hirugen.[44] Dysinosin A contains the same Adc subunit as that in oscillarin. The familiar perhydroindole core structure of dysinosin A harbors an additional hydroxy group at C5, resulting in a *trans* diaxial orientation. The N-terminal residues are D-Leu and a sulfated glyceric acid derivative not previously found in the other aeruginosins.

In 2003 the Pharmacia Corporation disclosed the isolation and characterization of a chlorinated dysinosin A derivative (**22**).[45] This peptide, here referred to as chlorodysinosin A,



was found to include 3-chloroleucine (Cleu), an amino acid unknown in the natural-product literature. The basic structure of **22** was deduced by NMR spectroscopy and degradation studies, and its structure and absolute configuration have recently been confirmed through total synthesis and a high-resolution X-ray structure with thrombin.[46]

Most recently, three new aeruginosins have been isolated from marine sponges of the family *Dysideidae*. Dysinosins B, C, and D (**23**–**25**)[36] are all from *Lamellodysidea chlorea*. They differ from dysinosin A in their substitution at C6 and the glyceric acid hydroxy groups. These peptides also incorporate a Val residue in place of the more common Leu. Like aeruginosins 205A and 205B, dysinosin B is a glycopeptide member of the aeruginosin family.

3. Biological Activity

The biological activities associated with the aeruginosin family of natural products have mainly been in relation to in vitro inhibitory activity against serine proteases (Table 1). Some of the aeruginosins have also been evaluated against other enzymes, such as the cysteine protease papain (Table 1). High inhibitory activities have been found against serine proteases with trypsin-like substrate specificity. These enzymes are involved in a number of important physiological processes, and their relevance in the complex blood coagulation cascade is well established.[47,48] Trypsin-like proteases are known to cleave substrates with positively charged amino acid residues in the P1 position. The aeruginosins, which contain a basic C-terminal arginine mimetic, are thus well equipped for accommodation within the active sites of these proteases.

3.1. Inhibition of Coagulation Cascade Factors

Blood coagulation is a complex process of ordered events involving both cellular (i.e., blood platelets and leukocytes) and proteinaceous (i.e., the coagulation factors and cofactors) components. The basic events involve platelet aggregation to form a primary platelet plug (primary hemostasis), followed by activation of plasma coagulation factors generating fibrin, which intertwines and reinforces the aggregated platelets,

Table 1: In vitro enzyme inhibitory activities of aeruginosins.

Compound	Enzyme IC_{50} [$\mu g\,mL^{-1}$][a]						
	thrombin	FVIIa	trypsin	plasmin	chymotrypsin	elastase	papain
aeruginosin 298A (**2**)[13,25]	0.3	–	1.0	>10	>10	>10	>10
aeruginosin 98A (**3**)[24,25]	7.0	–	0.6	6.0	>100	>100	100
aeruginosin 98B (**4**)[24,25]	10.0	–	0.6	7.0	>100	>100	100
aeruginosin 98C (**5**)[25]	3.3	–	3.9	5.0	>100	>100	–
aeruginosin 101 (**6**)[25]	3.2	–	3.0	3.3	>100	>100	–
aeruginosin 89A (**7**)[25]	0.03	–	0.4	0.02	>10	>10	>10
aeruginiosin 89B (**8**)[25]	0.05	–	6.6	0.46	>10	>10	>10
microcin SF608 (**9**)[28]	–	–	0.5	–	>20.0	–	–
aeruginosin 298B (**11**)[25]	>100	–	>100	>100	>100	>100	>100
aeruginosin EI461 (**13**)[29]	–	–	15% inh. at 45.5 $\mu g\,mL^{-1}$	0% inh. at 45.5 $\mu g\,mL^{-1}$	–	–	
aeruginosin 102A (**14**)[25,31]	0.04	–	0.2	0.3	>10	>10	>10
aeruginosin 102B (**15**)[25,31]	0.1	–	1.1	0.8	>10	>10	>10
aeruginosin 103A (**16**)[32]	9.0	–	51.0	68.0	–	–	–
aeruginosin 205A (**17**)[33]	1.5	–	0.07	–	–	–	–
aeruginosin 205B (**18**)[33]	0.17	–	0.07	–	–	–	–
oscillarin (**20**)[41,43]	0.018*	2.5*[b]	0.024*[b]	>260* (K_i)	–	–	–
dysinosin A (**21**)[46]	0.029*	0.206*	–	–	–	–	–
chlorodysinosin A (**22**)[46]	0.0038*	0.026*	0.025*	–	–	–	–
dysinosin B (**23**)[36]	0.13* (K_i)	0.07* (K_i)	–	–	–	–	–
dysinosin C (**24**)[36]	0.34* (K_i)	0.077* (K_i)	–	–	–	–	–
dysinosin D (**25**)[36]	>3.3* (K_i)	0.86* (K_i)	–	–	–	–	–

[a] IC_{50} and K_i values reported in μM were converted to $\mu g\,mL^{-1}$ and are indicated by an asterisk (*). [b] Unpublished IC_{50} values obtained in our laboratories. Courtesy of AstraZeneca, Mölndal, Sweden.

resulting in a strong fibrin clot (secondary hemostasis).[48–51] Two pathways are responsible for the biochemical coagulation cascade leading to the generation of fibrin: an intrinsic contact activation system, and an extrinsic tissue factor system (Figure 2).[48–51] Both pathways involve a stepwise activation of proteases, and they converge upon generation of active thrombin, a multifunctional serine protease,[52] which is responsible for the final cleavage of fibrinogen to fibrin. Positive-feedback mechanisms along both pathways accelerate the coagulation process. In recent years, the extrinsic system triggered by tissue factor has been considered as the major pathway to thrombinogenesis.[51]

Most of the coagulation factors are trypsin-like serine proteases.[48] Under normal physiological conditions the coagulation system is balanced by an anticoagulation system and a fibrinolysis system.[53,54] Imbalance between these mechanisms results in blood clotting or bleeding depending on which system dominates. A pathogenic imbalance in favor of the coagulation system may lead to thrombosis in humans.

Thrombosis and related complications are major causes of potentially fatal cardiovascular and cerebrovascular disease throughout the world.[55] Current anticoagulation therapies, such as the administration of heparins and coumarins, are limited by narrow therapeutic windows, severe side effects, and/or the need for parenteral administration. Intensive efforts have been made to develop new anticoagulants relying on direct inhibition of coagulation enzymes.[56–59] Thus, the knowledge that several of the aeruginosins exhibit high inhibitory potency against blood coagulation factors have made them attractive small-molecule targets in the search for

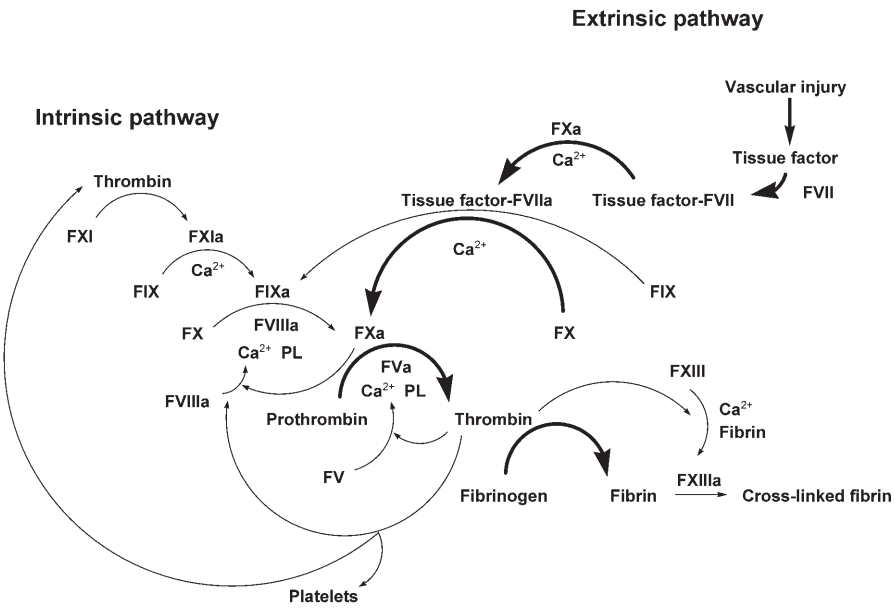


Figure 2. Schematic overview of the blood coagulation cascade.[51] The active forms of the factors and cofactors are denoted by an "a". PL refers to phospholipid, F to a factor.

new anticoagulants, although they may not be suitable as such for direct use in humans.

The central role of thrombin in the blood coagulation cascade has made it an attractive target for the development of antithrombotic drugs. Several X-ray crystal structures of the enzyme in complex with different inhibitors have been solved, providing important information about enzyme–inhibitor interactions.[60] Thrombin is a trypsin-like serine protease consisting of two polypeptide chains: A (36 residues) and B (259 residues) linked by disulfide bridges.[52] The most important regions of the thrombin active site for inhibition by compounds belonging to the aeruginosin family are the S1, S2, and the D-S3 subsites (Figure 3). The S1 subsite, also known as the specificity pocket, is characterized by an aspartic acid residue (Asp189) in the bottom of the pocket, which is able to recognize and engage in ionic interactions with inhibitors containing basic P1 side chains. An alanine residue in position 190 of the S1 pocket of thrombin distinguishes it from trypsin, which has a serine in this position.[61] The S1 subsite is also in close proximity to the oxyanion hole (the backbone amide NH groups of Ser195 and Gly193) and the Ser195-His57-Asp102 catalytic triad. The S2 subsite, or the proximal P pocket, is a hydrophobic pocket primarily created by Tyr60A and Trp60D in the Tyr60A-Pro60B-Pro60C-Trp60D thrombin insertion loop.[62,63] The distal D-S3 pocket (D pocket) is also hydrophobic but larger than S2, and is formed by the side chains of Leu99, Ile174, and Trp215. This pocket, which according to Schechter and Berger's nomenclature[64] is the S4 subsite, has been redefined as the D-S3 subsite based on the binding of D-phenylalanine in the X-ray crystal structure of the prototypical synthetic thrombin inhibitor D-Phe-Pro-Arg chloromethyl ketone (PPACK, Figure 3).[65] In addition to the aforementioned regions, hydrogen bonding to at least one of the Ser214-Trp215-Gly216 residues opposite the D-S3 subsite has been found to be important for inhibitory activity.[56,58,63]

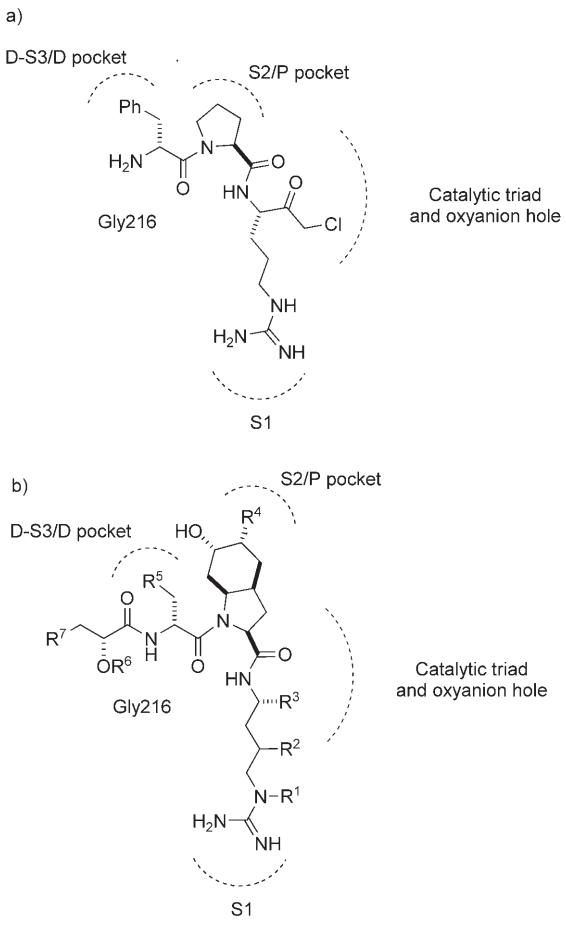


Figure 3. Schematic illustration of the thrombin active site bound to a) the D-Phe-Pro-Arg-mimicking inhibitor PPACK[65] and b) to the aeruginosins, as deduced from X-ray crystal structures.[20,43,44,46]

The most potent of the natural inhibitors of thrombin is the polypeptide hirudin ($K_i = 20$ fM).[66] Hirudin, a single-chain peptide 65 amino acids long, is derived from the salivary glands of the medicinal leech *Hirudo medicinalis*. It was isolated in the late 1950s and binds to thrombin in a bivalent fashion through interactions at both the active site and the remote "fibrinogen-binding exosite".[67] In general, the design of synthetic thrombin inhibitors has been based on the D-Phe-Pro-Arg sequence of PPACK, originally developed based on the cleavage sites of thrombin's natural substrates such as fibrinogen.[68] This sequence is closely mimicked by the general structure of the aeruginosins: a hydrophobic D-amino acid, a constrained bicyclic L-proline (Choi and OH-Choi), and a basic arginine-mimetic subunit.

As outlined in Table 1, all of the known aeruginosins except microcin SF608 and aeruginosin EI461 have been evaluated for inhibitory activity against thrombin. So far, four X-ray crystal structures of thrombin in complex with aeruginosin 298A (1A2C),[20] dysinosin A (Figure 4a),[44] oscillarin (1RIW, Figure 4b),[43] and chlorodysinosin A (2GDE, Figure 4c) have been solved.[46] The first crystal structure to be reported was that of thrombin in complex with aeruginosin 298A, which was solved in 1998 by Tulinsky and co-workers.[20] This structure showed an unexpected binding mode of the P3 leucine side chain located in the D-S3 subsite. The peculiar binding mode of the anticipated L-leucine at the time was later explained by a stereochemical revision from the L to D configuration.

More recently, the X-ray crystal structures of dysinosin A,[44] oscillarin,[43] and chlorodysinosin A,[46] in complex with thrombin have revealed binding modes similar to that observed for aeruginosin 298A. A schematic picture of the binding modes of the aeruginosins based on the four reported crystal structures is shown in Figure 3b. The binding mode of the aeruginosins has been found to closely resemble that of PPACK (Figure 3a) and other similar peptides: they form an antiparallel β strand with the main chain of Gly216 in the thrombin active site. The X-ray crystal structure of the chlorodysinosin A–thrombin complex revealed positional differences for the side chains of Glu192 and Arg173 compared to the dysinosin A–thrombin complex.[46]

Overall, the aeruginosins exhibit in vitro inhibitory activities against thrombin with micromolar to nanomolar IC_{50} values. The most potent of the aeruginosins isolated to date is chlorodysinosin A (thrombin $IC_{50} = 0.0057$ μM; 0.0038 μg mL^{-1}, Table 1). The basic P1 arginine mimetic seems to be essential for thrombin inhibitory activity. Aeruginosin 298B, which has the same molecular structure

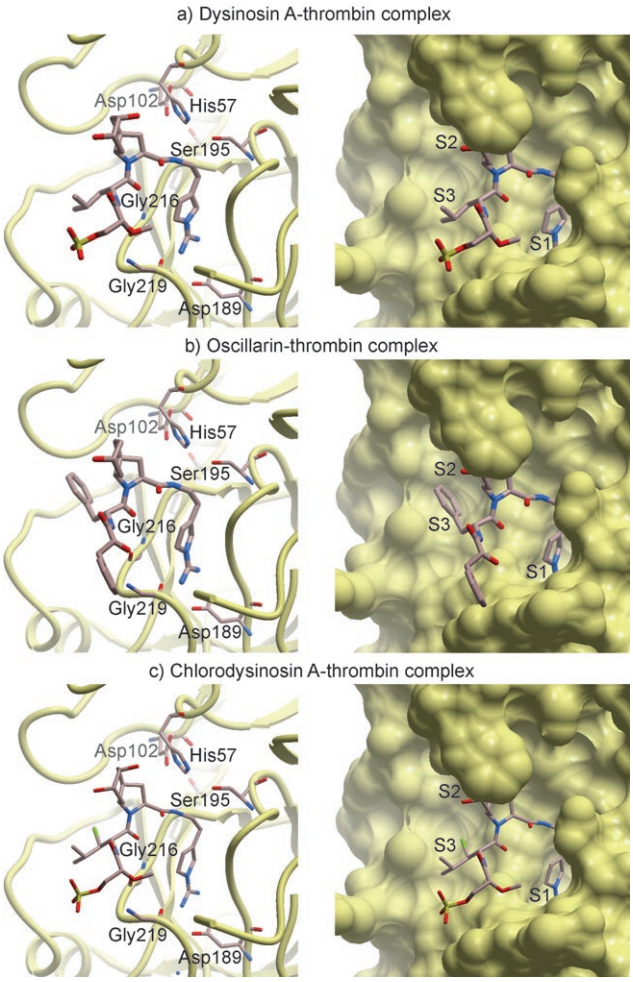


Figure 4. Different representations from X-ray crystal structures of thrombin in complex with dysinosin A,[44] oscillarin (1RIW),[43] and chlorodysinosin A (2GDE),[46] respectively, showing relevant amino acids in the active site. Ribbon diagrams (left) and Connolly surface representations (right) for thrombin in complex with dysinosin A (a), oscillarin (b), and chlorodysinosin A (c). Gray C, red O, blue N, yellow S, green Cl.

as aeruginosin 298A but lacks a basic P1 side chain, was completely inactive against thrombin ($IC_{50} > 100\ \mu g\,mL^{-1}$, Table 1).[25] In the reported X-ray crystal structures of aeruginosin 298A, dysinosin A, oscillarin, and chlorodysinosin A complexed with thrombin, the P1 side chain is found deeply buried in the S1 specificity pocket with the guanidine group forming a hydrogen-bonded salt bridge to the carboxylate of Asp189 (Figure 4a–c). Furthermore, the argininol hydroxy group of aeruginosin 298 A forms a hydrogen bond with the side chain of the catalytic His57.[20] Aeruginosins incorporating a P1 argininal have generally demonstrated stronger in vitro inhibition than those having an argininol or an agmatine side chain in the P1 position (cf. aeruginosins 89A/B, 102A/B versus aeruginosins 98A–C, 101, 205A/B, 298A, Table 1), illustrating the importance of an optimal P1 side chain.[25] The dysinosins and oscillarin have a unique 1-(*N*-amidino-Δ^3-pyrrolino)ethyl residue as a P1 arginine mimetic. A replacement of the five-membered cyclic Δ^3-pyrrolidino group in oscillarin by the originally proposed six-membered guanidine heterocycle resulted in a total loss of thrombin inhibitory activity.[43] This further supports the fundamental importance of a suitably oriented basic P1 subunit to attain high levels of in vitro thrombin inhibition.

According to the reported crystal structures of aeruginosin 298A,[20] oscillarin,[43] dysinosin A,[44] and chlorodysinosin A[46] with thrombin, the octahydroindole in P2 is positioned in the proximal S2 subsite interacting with Try60A and Trp60D. No hydrogen bonds between the 6-hydroxy group of Choi or the 5,6-hydroxy groups of the octahydroindole in OH-Choi and the thrombin active site are apparent. The octahydroindole core has been suggested as a subunit to better understand the selective binding to different enzymes. Sandler and co-workers[26] speculated that for the 6-*O*-sulfated aeruginosin 98B the selectivity for trypsin over thrombin might be due to the position of the sulfate group in a hydrophobic pocket in thrombin, whereas in trypsin the sulfate group projects out into solution. Furthermore, Carroll and co-workers[36] observed a decrease in selectivity between thrombin and FVIIa when a 6-*O*-glycosidic moiety is present in the dysinosins, suggesting this region to be further explored for selectivity between these two enzymes (cf. dysinosin B with dysinosins A and C, Table 1).

The P3 D-amino acid side chains of the aeruginosins have been found to occupy the hydrophobic D-S3 subsite in a similar manner to the D-phenylalanine in PPACK. Moreover, the backbone of the P3–P4 part of the aeruginosins binds to the backbone of the Gly216–Gly219 stretch in an antiparallel β-strand fashion (Figure 4a–c). The highly potent chlorodysinosin A, which differs from dysinosin A only by one chlorine atom in the β position of the D-leucine side chain, binds to thrombin in a way closely related to the binding of dysinosin A.[46] Only small differences in the orientation of a few of the enzyme active-site residues were observed, that is, Arg173 and Glu192. Neither the conformation of the leucine side chain nor the D-S3 protein binding site were altered in the X-ray structure of chlorodysinosin A (cf. Figure 4c). The higher inhibitory activity may originate from a more favorable charge distribution, increased hydrophobic interactions, a better accommodation of the chlorine atom in the D-S3 subsite, and a stabilization of the χ^1 dihedral angle of chloroleucine. This conformational stabilization was also supported by a short molecular dynamics simulation.[46] Additionally, it is likely that the binding of the chlorine-substituted P3 side chain is accompanied by the release of a water molecule, resulting in a gain in entropy.

Several of the most active aeruginosins possess an acidic sulfate group in the N-terminal P4 position (e.g. aeruginosins 89A/B, 102A/B, dysinosins A–C, and chlorodysinosin A, Table 1). In the thrombin–dysinosin A crystal structure this acidic group was shown to interact through several hydrogen bonds with two arginine residues (Arg173 and Arg221) on the surface of thrombin.[44] Since the desulfated dysinosin D demonstrated a 10-fold lower activity against thrombin than the sulfated dysinosins A–C, the P4 sulfate group was suggested to make a significant contribution to binding and potency.[36] However, oscillarin, which is devoid of a P4 sulfate, still shows even higher inhibitory potency against

thrombin. In fact, no interactions between the terminal P4 phenyl group of oscillarin and thrombin were observed in the X-ray crystal structure (Figure 4b).[43]

Attention has been directed towards other coagulation cascade factors besides thrombin (FIIa). To the best of our knowledge, only the dysinosins and oscillarin have been examined for inhibition of coagulation factors other than thrombin, such as factor VIIa and factor Xa (Table 1). In a factor Xa assay chlorodysinosin A and dysinosin A had IC_{50} values of 1.54 μM and 5 μM, respectively.[46]

Although no X-ray crystal structure of the aeruginosins in complex with factor VIIa is available, the high homology of the active site with that found in thrombin suggests a similar binding mode.[69,70] As mentioned above, Carroll and co-workers[36] reported that the presence of a sugar residue on the octahydroindole core as in dysinosin B had an influence on the selectivity between FVIIa and thrombin. This sugar moiety also resulted in a slight increase in affinity for FVIIa (cf. dysinosin B with dysinosins A and C, Table 1). Moreover, they found that an N-terminal P4 sulfate group seems to contribute to both thrombin and FVIIa binding (cf. dysinosin D with dysinosins A–C, Table 1).[36] In accordance with this observation, oscillarin, which lacks a P4 sulfate, exhibits a weak activity against FVIIa ($IC_{50} = 3.9$ μM, Table 1). Since oscillarin still shows a high activity against thrombin, a P4 sulfate group might be more important for inhibition of FVIIa.

Chlorodysinosin A exhibits the highest inhibitory activity against FVIIa among all the aeruginosins ($IC_{50} = 0.039$ μM, Table 1).[46] The large difference in activity between chlorodysinosin A and dysinosin A indicates a positive effect of the P3 chlorine atom upon binding to FVIIa, similar to that observed upon the binding to thrombin.

3.2. Inhibition of Trypsin

Trypsin is a digestive enzyme frequently employed as a marker for inhibition of trypsin-like serine proteases. Inhibition of trypsin might also by itself be of interest for the treatment of pancreatic disorders, such as pancreatitis.[71] The structure of aeruginosin 98B cocrystallized in complex with trypsin was solved in 1998 by Sandler and co-workers.[26] The most striking feature was the lack of interactions between aeruginosin 98B and the trypsin catalytic triad (Ser195-His57-Asp102), indicating a new possible mode of serine protease inhibition. Apart from this structure, only one partially solved structure of trypsin in complex with oscillarin has been reported in a patent from Boehringer Mannheim GmbH in 1997.[42] In general, the binding of aeruginosin 98B to trypsin is similar to that shown for the aeruginosins in complex with thrombin and closely resembles that of a D-Phe-Pro-Arg tripeptide.[72] The basic P1 agmatine side chain is buried in the S1 specificity pocket, forming two strong hydrogen bonds to the side-chain carboxylate of Asp189. The Choi moiety is situated in the S2 subsite with the 6-*O*-sulfate group reaching out into solution. It was speculated that the Choi sulfate group might be the primary determinant for selectivity for trypsin over thrombin.[26] The P3 D-*allo*Ile side chain is located in the Trp215 "aryl-binding site" and makes van der Waals interactions with Trp215 and Leu99. If the stereochemistry of the D-*allo*Ile was changed to L-Ile or L-*allo*Ile, the P3 side chain would sterically interfere with the Choi sulfate group. Furthermore, antiparallel β-strand binding is observed between the backbone of D-*allo*Ile and Gly216. The phenol hydroxy group utilizes a water molecule in the P4 position, also observed in crystal structures of uncomplexed trypsin,[73] as a bridge to interact with Cys220 and Ser146.

Most of the aeruginosins have been assessed for trypsin inhibitory activity (Table 1). Not surprisingly, the structure–activity relationships (SARs) for trypsin are similar to those for the trypsin-like serine protease thrombin. To date, the most potent natural aeruginosins are chlorodysinosin A and oscillarin, both demonstrating IC_{50} values against trypsin of 0.037 μM (0.025 and 0.024 $\mu g\,mL^{-1}$, respectively, Table 1). In accordance with the substrate preference of trypsin, a basic P1 end group appears to be critical for inhibition. Aeruginosin 298B, which lacks a basic P1 group, is inactive against trypsin ($IC_{50} > 100\ \mu g\,mL^{-1}$, Table 1).[25] Moreover, Kodani and co-workers[32] suggested the bulky P1 ethyl hemiaminal of aeruginosin 103A to be responsible for its low trypsin inhibitory potency ($IC_{50} = 51.0\ \mu g\,mL^{-1}$, Table 1). Fukuta and co-workers[74] suggested that the difference in trypsin potency between aeruginosin 298A and 102A might be due to the P4 sulfate group found in the more potent 102A ($IC_{50} =$ 1.0 and 0.2 $\mu g\,mL^{-1}$, respectively, Table 1).

3.3. Other Biological Activities

The aeruginosins have been evaluated for activity against a number of enzyme targets (Table 1). Plasmin, a trypsin-like serine protease involved in the fibrinolytic system (see Section 3.1), is inhibited by several of the naturally occurring aeruginosins. Plasmin has also been implicated in the angiogenesis and metastasis during the progression of cancer, which has made it a potential target for development of anticancer agents.[75] The most potent plasmin inhibitor of the aeruginosin family reported to date is aeruginosin 89A ($IC_{50} = 0.02\ \mu g\,mL^{-1}$, Table 1).

So far, no inhibitory activity of the aeruginosins against the digestive serine proteases chymotrypsin and elastase has been found (Table 1). Ishida and co-workers[25] tested some of the aeruginosins against the cysteine protease papain (aeruginosins 89A/B, 102A/B, 98A/B, and 298A/B, Table 1). However, only weak activity of aeruginosins 98A and 98B was demonstrated, both registering IC_{50} values of 100 $\mu g\,mL^{-1}$. Microcin SF608 is the only aeruginosin that has been evaluated against the metalloprotease neprolysine, but no inhibition was detected at 20.0 $\mu g\,mL^{-1}$.[28]

4. Total Syntheses

4.1. Aeruginosins 298A and 298B

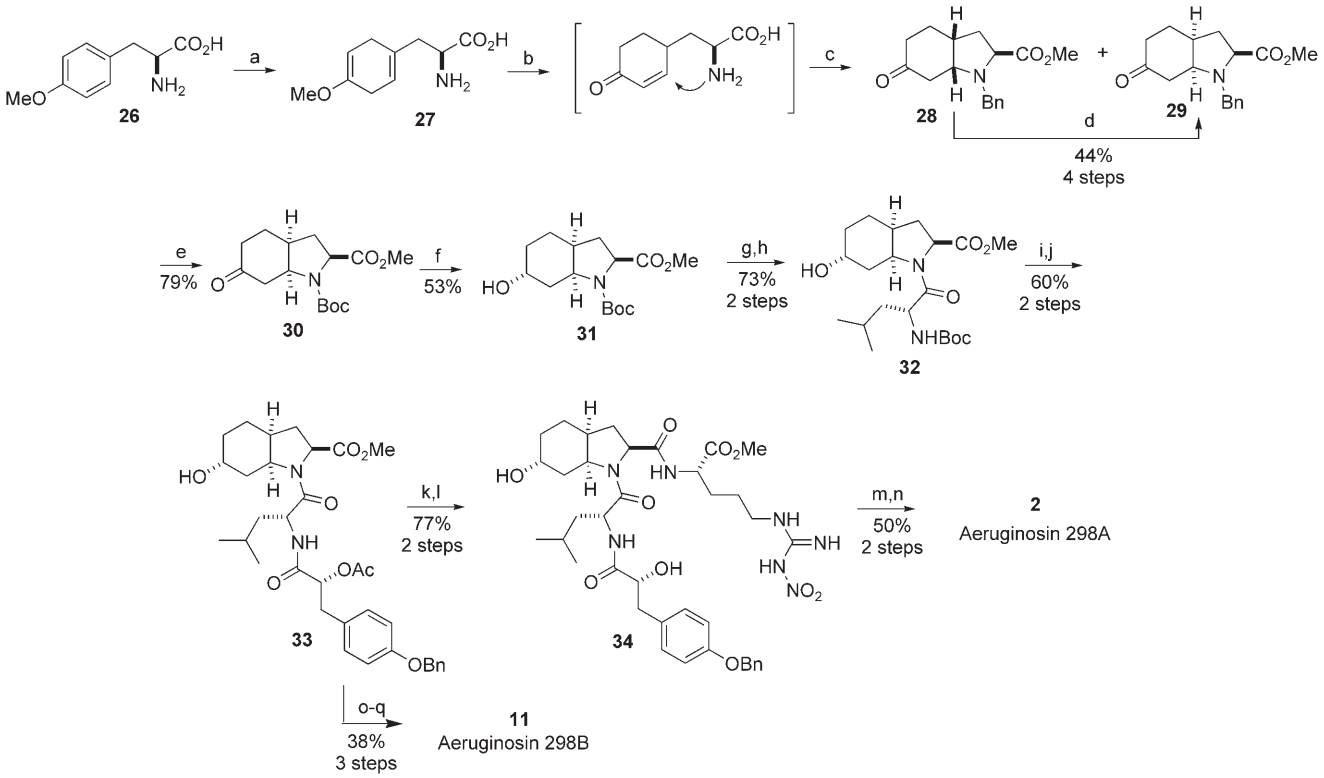
The isolation of aeruginosin 298A by Murakami et al.[13] in 1994 was followed some years later by an X-ray crystal

structure of the natural product bound to hirugen–thrombin.[20]

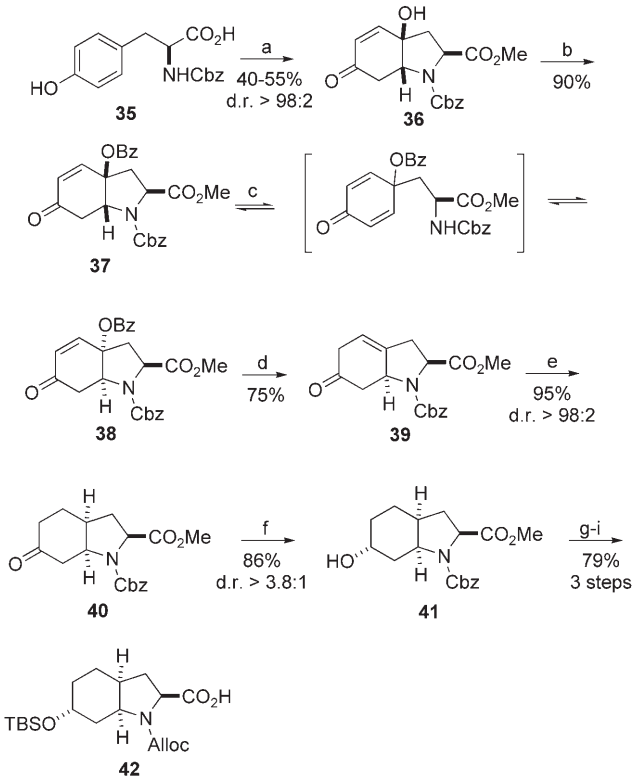
The absolute configuration obtained from the crystal structure served as the basis for two total syntheses reported in 2000. Starting from L-Tyr(OMe)-OH (**26**), Bonjoch and co-workers[76] described an efficient entry into the (2*S*,3a*S*,6*R*,7a*S*)-6-hydroxyoctahydroindole-2-carboxylic acid (L-Choi) motif found in many of the aeruginosins. This method was featured in the first total synthesis of aeruginosins 298A[21] and 298B.[22] The azabicyclic core subunit was obtained in two steps by Birch reduction of **26** followed by acidic cleavage of the enol ether and Michael-type addition of the pendant amine nucleophile (Scheme 1). The resulting secondary amine was treated directly with BnBr to afford a mixture of isomers **28** and **29**. Thermodynamic equilibration of this mixture in the presence of MeOH/aq. 8 N HCl provided the more stable isomer **29** in 44% overall yield from **26**. For the purpose of completing the synthesis of aeruginosins 298A and 298B, intermediate **29** was converted into **31**. A variety of ketone-reduction conditions were examined in an effort to secure the desired 6*R* stereochemistry of the alcohol. Optimal selectivity in favor of the 6*R* isomer (8:1) was achieved by reduction with LS-Selectride. With the protected L-Choi derivative **31** in hand, aeruginosin 298A was assembled using standard peptide-coupling techniques. Thus, Boc removal was followed by BOP-mediated coupling with Boc-D-Leu-OH to give dipeptide **32** in 73% yield. Following the same protocol, the protected hydroxyphenyllactic acid unit, prepared according to Wang's procedure,[77] was installed prior to hydrolysis of the methyl ester and coupling to L-Arg(NO_2)-OMe·HCl to give the fully assembled precursor **34**. Reduction with $LiBH_4$ and removal of the protecting groups by hydrogenolysis afforded aeruginosin 298A (**2**).

It is important to note that the synthesis of the proposed structure of aeruginosin 298A, in which an L-Leu amide subunit is present, was first completed by Bonjoch and co-workers[21] following the assignment derived from the X-ray structure of the ternary complex.[20] The discrepancy observed between the ^{1}H NMR spectra of this isomeric product (**1**) and that reported for the natural product led to the synthesis and structural revision of aeruginosin 298A (**2**), now containing D-Leu. Based on these findings, Bonjoch and co-workers[22] also prepared aeruginosin 298B (**11**) from **33**, again confirming the presence of a D-Leu amide subunit.

In contrast to the tyrosine-reduction route reported by the Bonjoch group, Wipf and co-workers[23] developed a tyrosine-oxidation route toward the key Choi bicyclic hydroindole, which is present in the aeruginosins as well as other alkaloidal natural products (Scheme 2). Treatment of Cbz-L-Tyr-OH (**35**) with $PhI(OAc)_2$ followed by exposure to methanol/$NaHCO_3$ gave **36** with a diastereoselectivity of d.r. > 98:2. As with the tyrosine-reduction route, access to the desired stereochemistry at the ring-junction carbons was possible by the thermodynamic equilibration of benzoylated derivative **37** to give the more stable isomer **38**; this proceeded by ring-opening to the cyclohexadienone followed by Michael-type



Scheme 1. Synthesis of aeruginosin 298A by Bonjoch et al. a) Li, NH_3, THF/*t*BuOH, −78 °C; b) MeOH, 7.5 N HCl, 35 °C; c) BnBr, $NaHCO_3$, EtOH, 70 °C; d) MeOH, 8 N HCl, 65 °C; e) H_2, $Pd(OH)_2$, Boc_2O, EtOAc; f) LS-Selectride, THF, −78 °C; g) TFA, CH_2Cl_2, 0 °C; h) Boc-D-Leu, BOP, NMM, CH_2Cl_2; i) TFA, CH_2Cl_2, 0 °C; j) (*O*-Bn,*O*-Ac)-D-Hpla, BOP, NMM, CH_2Cl_2; k) 0.1 N LiOH/THF; l) L-Arg(NO_2)-OMe, BOP, NMM, DMF; m) $LiBH_4$, THF; n) H_2, Pd/C, EtOAc/MeOH, 6 N HCl, 1 atm; o) 0.1 N LiOH/THF; p) NH_4OH, PyBOP; q) H_2, Pd/C.

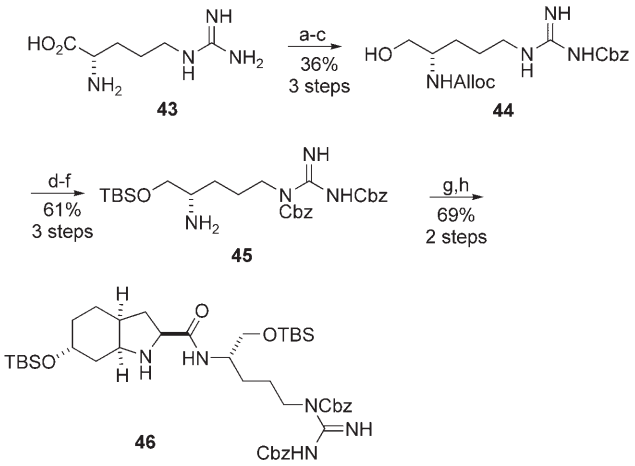


Scheme 2. Synthesis of aeruginosin 298A by Wipf et al. Synthesis of L-Choi derivative **42**. a) $PhI(OAc)_2$, $NaHCO_3$, MeOH; b) Bz_2O, DMAP, pyridine, CH_2Cl_2; c) $NaHCO_3$, DMSO, 90 °C; d) Zn dust, AcOH/THF, 65 °C; e) H_2, 5% PtO_2, 10% AcOH/EtOH, 0 °C; f) L-Selectride, THF, −78 °C; g) TBSOTf, ImH, CH_2Cl_2; h) H_2, Pd/C, EtOH, then AllocCl, pyridine; i) LiOH, THF/H_2O, 40 °C.

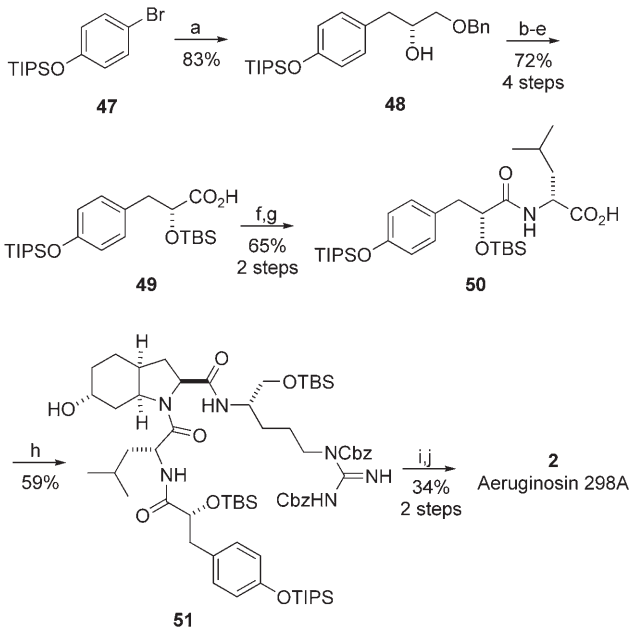
closure. The resulting 1.6:1 mixture of products was then separated to give **38** in 78% yield after recycling of recovered **37**. Reductive displacement of the benzoate ester using activated Zn dust or SmI_2 gave the β,γ-unsaturated ketone **39**, which was hydrogenated to give *cis*-fused azabicyclic ketone **40**. Reduction of the ketone function with L-Selectride afforded a 3.8:1 mixture of products having *exo* and *endo* hydroxy groups. Protection of the major product (**41**) as a TBS ether, followed by exchange of the Cbz protecting group for Alloc, and cleavage of the methyl ester then afforded Choi derivative **42**.

The Argol fragment was prepared from L-Arg-OH (**43**) by sequential protection of the α-amine and guanidine groups, followed by reduction to the primary alcohol **44** (Scheme 3). Protecting-group manipulation gave **45** in 22% overall yield from arginine. The Choi-Argol fragment was obtained by pentafluorophenyl ester-mediated coupling of amine **45** to acid **42** in 69% yield. Removal of the Alloc group under normal conditions gave subunit **46**.

The synthesis of the appropriately protected Hpla-Leu fragment and final assembly of aeruginosin 298A by Wipf and co-workers[23] is depicted in Scheme 4. The Hpla subunit was prepared by reaction of (*R*)-benzylglycidol with an arylcuprate to give **48**. Protection of the alcohol as the TBS ether was followed by hydrogenolysis of the benzyl ether and oxidation of the resulting alcohol to carboxylic acid **49**. Coupling to D-



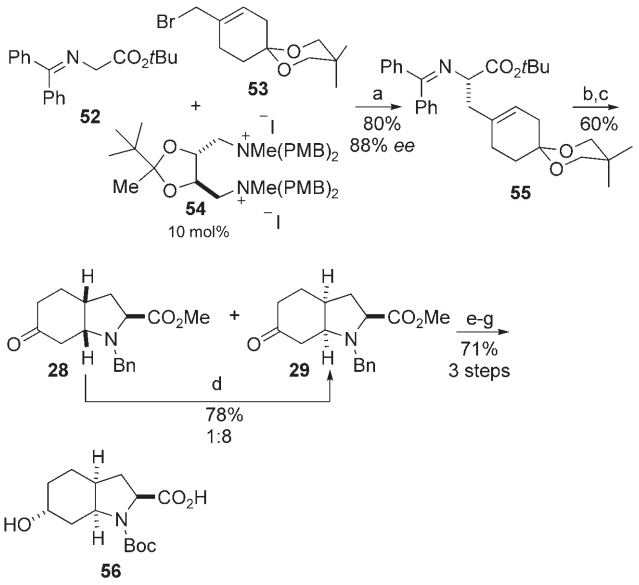
Scheme 3. Synthesis of aeruginosin 298A by Wipf et al. Synthesis of amide **46**. a) AllocCl, aq. NaOH; b) CbzCl, aq. NaOH, THF; c) IBCF, NMM, DMF, −20 °C, then $NaBH_4$; d) TBSOTf, ImH, CH_2Cl_2; e) CbzCl, DMAP, K_2CO_3, DMF; f) Bu_3SnH, $[Pd(Ph_3)_4]$, AcOH, THF; g) **42**, FDPP, DIEA, CH_2Cl_2; h) Bu_3SnH, $[Pd(PPh_3)_4]$, AcOH, THF.



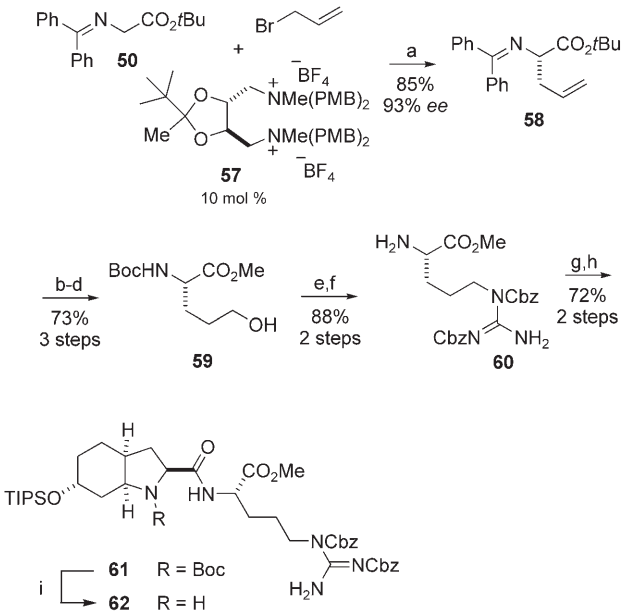
Scheme 4. Synthesis of aeruginosin 298A by Wipf et al. Completion of the synthesis. a) *t*BuLi, $CuBr{\cdot}SMe_2$, THF, −78 → °C to −45 °C then (*R*)-benzylglycidol, $BF_3{\cdot}Et_2O$, −45 °C to −20 °C; b) TBSOTf, ImH, CH_2Cl_2; c) H_2, Pd/C, EtOAc; d) DMP, CH_2Cl_2; e) $NaClO_2$, NaH_2PO_4, 2-methyl-2-butene, *t*BuOH/H_2O; f) L-Leu-OBn, DEPC, DIEA, CH_2Cl_2; g) H_2, Pd/C, EtOH; h) **46**, DEPBT, DIEA, CH_2Cl_2; i) aq. HF; j) H_2, Pd/C, EtOH.

Leu-OBn, and ester cleavage by hydrogenolysis gave **50**. Fragments **50** and **46** were then joined in the presence of DEPBT[23,78,79] to give intermediate **51** in 59% yield. Removal of the silyl and Cbz protecting groups afforded aeruginosin 298A (**2**).

More recently, Shibasaki and co-workers[74,80] devised a synthesis of the L-Choi subunit by application of catalytic asymmetric phase-transfer alkylation reactions (Scheme 5). The enolate of glycine derivative **52** was previously shown to



Scheme 5. Synthesis of aeruginosin 298A by Shibasaki et al. Synthesis of L-Choi derivative **56**. a) $CsOH{\cdot}H_2O$, toluene/CH_2Cl_2 (7:3), −70 °C; b) 4 N HCl, MeOH; c) BnBr, $NaHCO_3$, EtOH; d) conc. HCl; e) H_2, Pd/C, Boc_2O; f) LS-Selectride, THF, −78 °C; g) aq. LiOH/MeOH.



Scheme 6. Synthesis of aeruginosin 298A by Shibasaki et al. Synthesis of fragment **62**. a) $CsOH{\cdot}H_2O$, toluene/CH_2Cl_2 (7:3); b) HCl; c) Boc_2O, TEA; d) 9-BBN, then H_2O_2; e) DEAD, PPh_3, di-Cbz-guanidine; f) *p*-TsOH; g) **56**, EDC, HOBt; h) TIPSOTf, DIEA; i) $ZnBr_2$.

react with a variety of electrophiles to give α-amino acids of high enantiomeric purity.[81] For the synthesis of the Choi core of aeruginosin 298A, the enolate of **52** was alkylated with bromide **53** in the presence of 10 mol% **54** as a catalyst to give **55** in 80% yield and 88% *ee*. Ketal deprotection and subsequent intramolecular Michael-type addition then afforded bicyclic ketones **28** and **29**, which were converted to **56** following the method previously described by Bonjoch and co-workers.[22]

A synthesis of the L-Argol subunit utilizing asymmetric phase-transfer alkylation methodology is shown in Scheme 6. Thus the allylglycine precursor **58** was obtained in excellent yield and enantioselectivity. Functional-group manipulation led to alcohol **59**, which was further transformed into guanidine intermediate **60**. Peptide coupling with **56** gave protected L-Choi-Argal fragment **61** in 72% yield. Boc removal then afforded compound **62**.

The Hpla subunit was prepared by asymmetric epoxidation of imidazolide **63** (Scheme 7).[82] The resulting peroxyester **64** could then be coupled by simple mixing with D-4,5-dehydroleucine-O*t*Bu to give amide **65**. Reduction of the double bond by catalytic hydrogenation, protection of the alcohol, and cleavage of the *t*-butyl ester then gave the Hpla-Leu fragment **66**. After extensive optimization, the final condensation reaction was performed in the presence of HATU to give **67** in 54% yield with minimal racemization of the Leu amide subunit (< 5%). Finally, removal of the methyl ester and further deprotection afforded aeruginosin 298A (**2**). Although the Argol and Leu subunits of the natural product are easily accessible from readily available chiral starting materials, Shibasaki's phase-transfer alkylation methodology proved particularly useful for the synthesis of analogues of aeruginosin 298A featuring unnatural amino acid residues.[74]

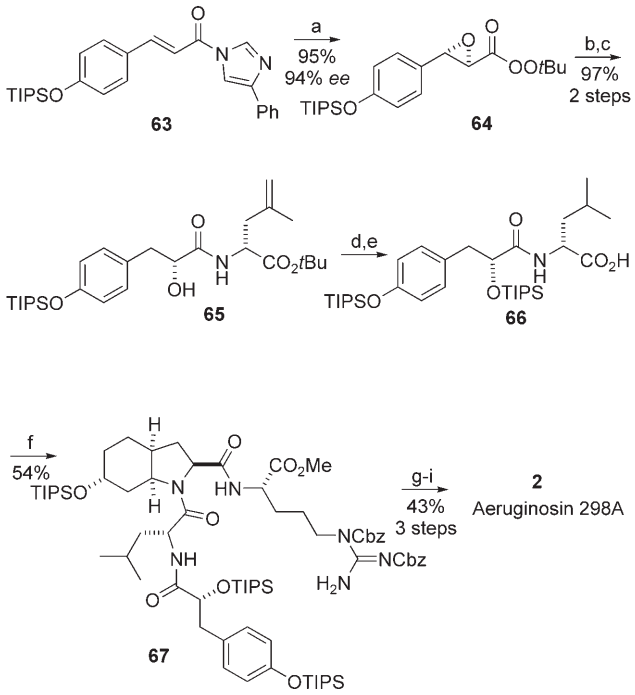
4.2. Microcin SF608

Having secured an entry into the bicyclic L-Choi residue from L-tyrosine utilizing a reductive transformation (Scheme 1), Bonjoch and co-workers[83] applied this methodology to the total synthesis of other aeruginosin peptides. In the case of the trypsin inhibitor microcin SF608, a new preparation of the Hpla subunit, relying on diazotation of Tyr derivative **68**, was also developed (Scheme 8).[83] Starting from Choi derivative **31**, sequential couplings mediated by PyBOP first afforded intermediate **70** in high yield, which was further extended to include the Hpla subunit, affording the protected precursor **71**. Final deprotection afforded microcin SF608 (**9**) in excellent overall yield from **70**.

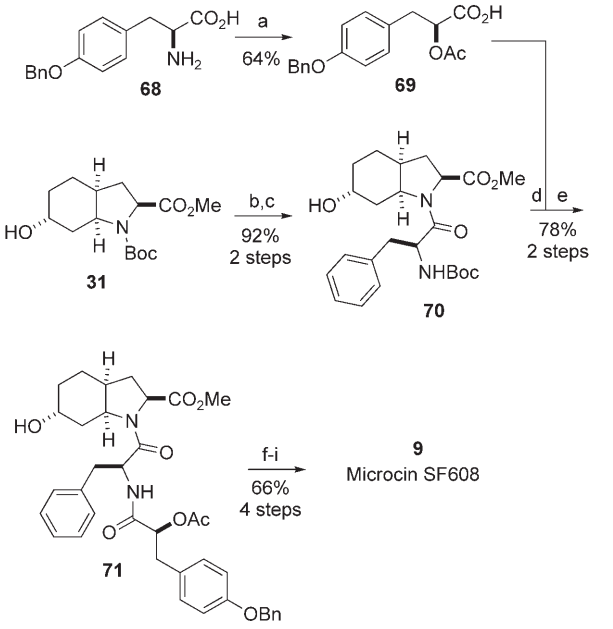
Interestingly, microcin SF608 showed two peaks by analytical RP-HPLC and two sets of resonances in its ^{1}H NMR spectrum. The observed 3:1 ratio of products was attributed to an unusually slow *cis–trans* rotational isomerism about the Choi-Phe amide bond. A pattern of H-2 and H-7a chemical-shift differences between the *cis* and *trans* rotamers of **9** and its precursors provides a reliable method to determine the relative configuration and rotational conformation of the Xaa-Choi dipeptide core of the aeruginosins.

4.3. Aeruginosin EI461

Bonjoch and co-workers[30] utilized the previously undesired Choi isomer **28** as a precursor for the synthesis of the core of aeruginosin EI461. In constrast to the other aeruginosins, in EI461 the hydrogens at the octahydroindole ring junction have a *syn* relationship to the methyl ester substituent (Scheme 9). Diastereoselective reduction of the keto

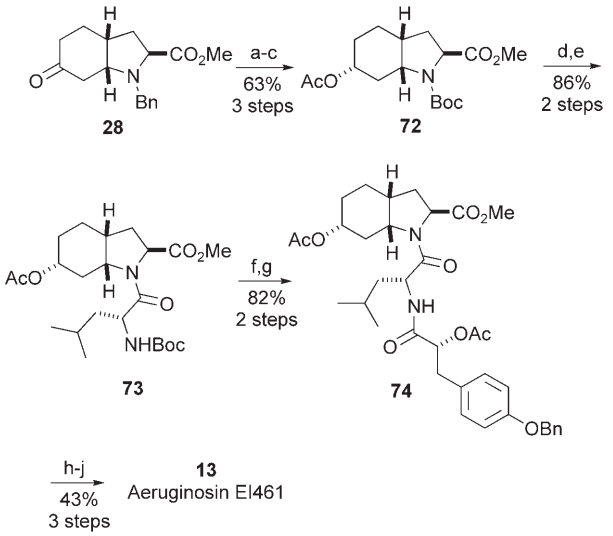


Scheme 7. Synthesis of aeruginosin 298A by Shibasaki et al. Completion of the synthesis. a) La-(*S*)-BINOL-PPh_3=O (10 mol%), TBHP, MS 4Å, THF; b) D-4,5-dehydroleucine-O*t*Bu, THF; c) H_2, Pd/C; d) TIPSOTf, DIEA; e) TMSOTf; f) **62**, HATU, DIEA, CH_2Cl_2; g) $LiBH_4$, THF; h) HF·py; i) H_2, Pd/C.



Scheme 8. Synthesis of microcin SF608 by Bonjoch et al. a) *i*-$C_5H_{11}ONO$, AcOH, NaOAc; b) TFA, CH_2Cl_2, 0°C; c) Boc-L-Phe-OH, PyBOP, NMM, CH_2Cl_2; d) TFA, CH_2Cl_2, 0°C; e) PyBOP, NMM, CH_2Cl_2; f) 0.1 N LiOH, THF; g) di-Boc-Agma, PyBOP, NMM, DMF; h) 6 N HCl, MeCN; (i) H_2, Pd/C.

function in **28** with $NaBH_4$ followed by protecting-group interconversion afforded the 3a,7a-*diepi*-L-Choi derivative **72** in 63% yield. Attachment of the *N*-Boc-D-Leu residue and



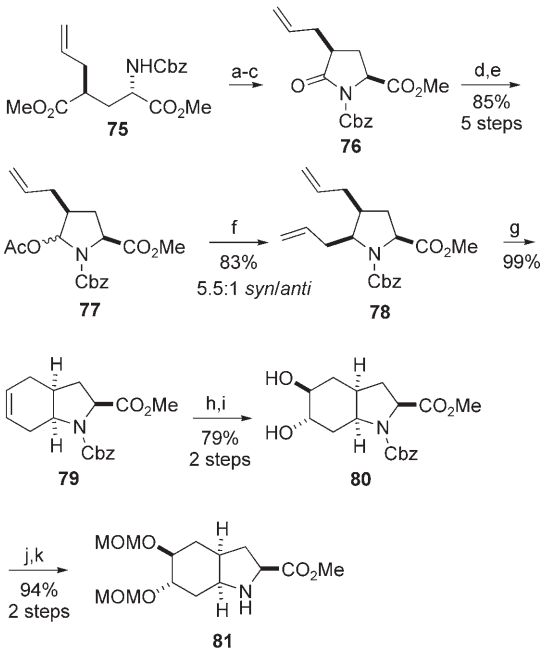
Scheme 9. Synthesis of aeruginosin EI461 by Bonjoch et al. a) $NaBH_4$; b) H_2, $Pd(OH)_2$/C, Boc_2O; c) Ac_2O; d) TFA, CH_2Cl_2, 0°C; e) Boc-D-Leu, CH_2Cl_2, PyBOP, NMM; f) TFA, CH_2Cl_2, 0°C; g) **69**, PyBOP, NMM, CH_2Cl_2; h) 0.1 N LiOH, THF; i) HOBt, EDC, NMM, THF then NH_4OH; j) H_2, Pd/C.

further extension with the Hpla subunit then gave aeruginosin EI461 in 19% overall yield from **28**. The synthesis of aeruginosin EI461 (**12**) led to the revision of the originally proposed structure, which featured the more common *endo* methoxycarbonyl substituent. Bonjoch and co-workers[30] also synthesized **12** starting from Choi derivative **30**, confirming the original structural misassignment.

4.4. Dysinosin A

The use of asymmetric alkylation methodology and of tyrosine as a chiral progenitor proved to be practical solutions to the synthesis of the azabicyclic Choi core structures of the aeruginosins. In their efforts toward the potent thrombin and factor VIIa inhibitor dysinosin A, Hanessian and co-workers[84] developed a conceptually distinct strategy towards the requisite hydroxy-Choi residue relying on *N*-acyl iminium ion alkylation and ring-closing metathesis (Scheme 10). In this approach, the (4*S*)-allylglutamate precursor **75**, which is readily available in high yield from L-glutamic acid,[85] was first converted into C4-substituted pyroglutamate **76**, thereby securing a *cis* relationship of both substituents. Reduction of the lactam function with Super-hydride ($LiBHEt_3$)and O-acetylation of the intermediate hemiaminal provided **77** in 85% yield. After extensive screening of various solvents, Lewis acids, and N-protecting groups, it was found that C5-allylation of the *N*-acyl iminium ion intermediate could be achieved using allyltributylstannane and $BF_3{\cdot}Et_2O$ in toluene to give the *syn*-diallyl-substituted proline derivative **78** in 83% yield and 5.5:1 diastereoselectivity. Interestingly, the *N*-Boc-protected derivative afforded the diallyl product with a 1:2 diastereomeric ratio in favor of the undesired *anti* isomer.

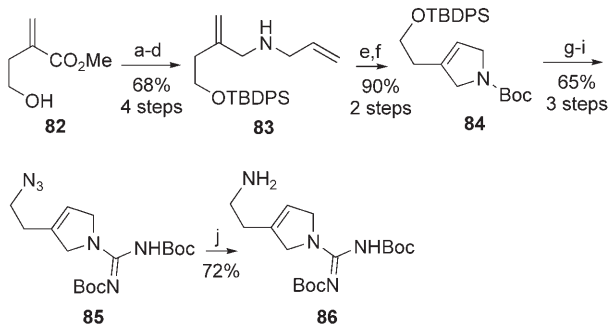
Treatment of **78** with Grubbs' first generation catalyst[86] in refluxing dichloromethane furnished bicyclic alkene **79**,



Scheme 10. Synthesis of dysinosin A by Hanessian et al. Synthesis of the hydroxy-L-Choi derivative **81**. a) TFA, CH_2Cl_2; b) Toluene, reflux; c) LHMDS, CbzCl, THF, −78 °C; d) $LiHBEt_3$, THF, −78 °C; e) Ac_2O, DMAP, CH_2Cl_2; f) $BF_3{\cdot}Et_2O$, allyltributylstannane, toluene, −78 °C; g) 1 mol% Grubbs' first generation catalyst, CH_2Cl_2, reflux; h) *m*CPBA, CH_2Cl_2; i) cat. TFA, THF/H_2O; j) MOMCl, DIEA, CH_2Cl_2; k) H_2, Pd/C, MeOH.

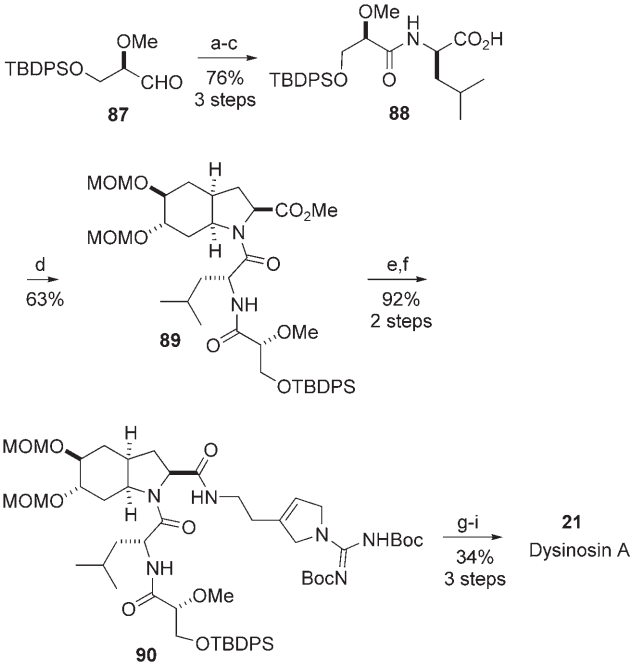
which was transformed into *trans* diaxial diol **80** by a face-selective epoxidation and acid-catalyzed opening with aqueous trifluoroacetic acid. Protecting-group manipulation gave 5-hydroxy-L-Choi derivative **81** in 44% overall yield from **75**.

In addition to featuring a novel dihydroxylated core structure, dysinosin A also contains an unusual 1-(*N*-amidino-Δ^3-pyrrolino)ethyl (Adc) subunit as a cyclic P1 arginine mimetic. The synthesis of this subunit is depicted in Scheme 11. The known hydroxyester **82** was converted to the diallyl intermediate **83**, which was then subjected to a ring-closing metathesis reaction using Grubbs' first generation catalyst to give **84** in high yield. Cleavage of the TBDPS ether was followed by introduction of azide by a Mitsunobu reaction, and the Boc group was cleaved to give the Δ^3-pyrroline.[84] Guanidinylation using the Goodman reagent[87] provided **85**, which was reduced to give protected Adc derivative **86** in 28% overall yield from **82**.



Scheme 11. Synthesis of dysinosin A by Hanessian et al. Synthesis of the Adc subunit. a) TBDPSCl, ImH, DMF; b) DIBAL-H, CH_2Cl_2; c) MsCl, NEt_3, CH_2Cl_2; d) allylamine, CH_2Cl_2; e) Boc_2O, NEt_3, CH_2Cl_2; f) Grubbs' first generation catalyst, CH_2Cl_2; g) TBAF, THF; h) PPh_3, DEAD, $(PhO)_2P(O)N_3$, THF; i) TFA, CH_2Cl_2, then di-*N*-Boc-triflylguanidine, NEt_3; j) PPh_3, THF/H_2O.

The final assembly of dysinosin A is shown in Scheme 12. Thus, oxidation of aldehyde **87**, readily available from D-mannitol,[88] and coupling of the resulting carboxylic acid to D-Leu-OBn, gave **88** after hydrolysis of the benzyl ester. The



Scheme 12. Synthesis of dysinosin A by Hanessian et al. Completion of the synthesis. a) $NaClO_2$, 2-methyl-2-butene, NaH_2PO_4, *t*BuOH; b) D-Leu-OBn, EDC, HOBt, CH_2Cl_2; c) H_2, Pd/C, MeOH; d) **81**, BOPCl, DIEA, MeCN; e) LiOH, THF/H_2O; f) **86**, EDC, HOBt, CH_2Cl_2; g) TBAF, THF; h) $SO_3{\cdot}py$, Bu_2SnO, CH_2Cl_2; i) TFA, CH_2Cl_2 then RP-HPLC.

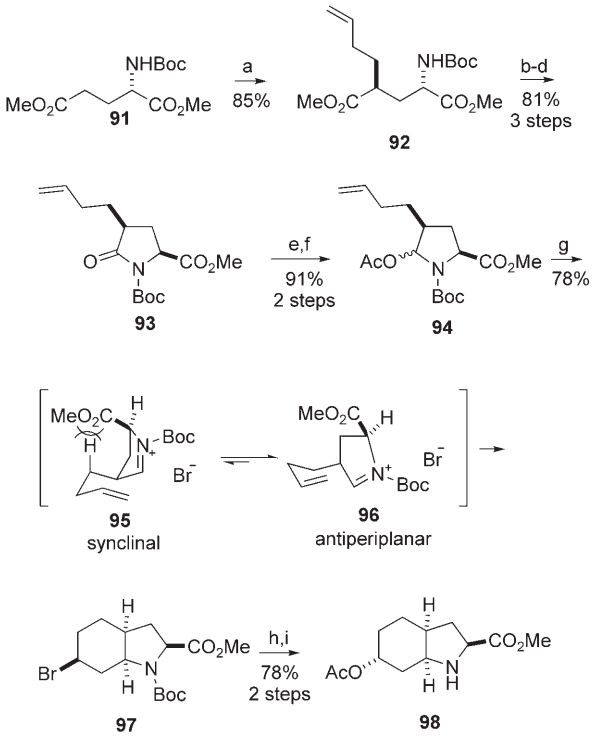
critical coupling step between amine **81** and carboxylic acid **88** proceeded in 63% yield in the presence of BOPCl. Ester hydrolysis and coupling of the Adc subunit gave **90.** Cleavage of the TBDPS group and sulfation of the resulting alcohol was achieved in the presence of dibutyltin oxide as an activator.[89,90] Acidic deprotection and purification by RP-HPLC afforded dysinosin A (**21**). The first synthesis of dysinosin A confirmed the structure of the natural product and provided an efficient entry into the novel 5-hydroxy-L-Choi and Adc structural motifs.

4.5. Oscillarin

In a 1996 patent, scientists at Boehringer Mannheim GmbH disclosed the structure of a new aeruginosin containing a hitherto unknown basic P_1 subunit to which the name oscillarin was given (**19**).[41] After a total synthesis of **19** by Hanessian et al.[43] it became evident that the structure of the

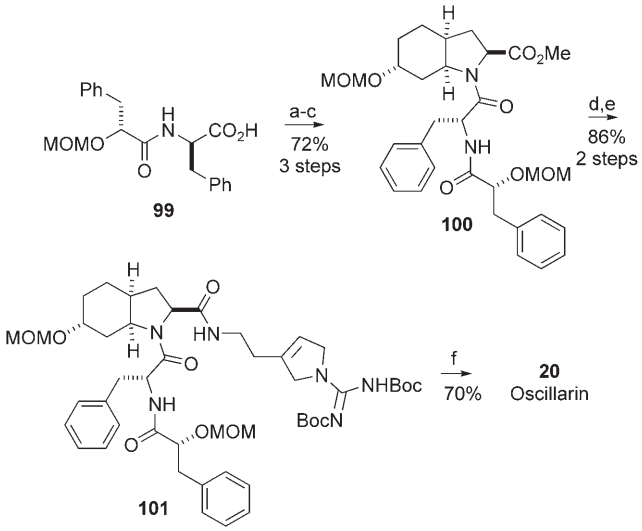
P1 subunit had been incorrectly assigned. A second patent issued in 1997 depicted the Δ^3-pyrroline moiety found in dysinosin A as the true P1 subunit.[42] In their approach to oscillarin, Hanessian and co-workers sought to again apply *N*-acyl iminium ion chemistry to a general construction of the octahydroindole motif. The total synthesis and subsequent structural revision of oscillarin provided a platform for the development of azonia-Prins halocarbocyclizations en route to the L-Choi residue and its derivatives.[43,91]

As shown in Scheme 13, diastereoselective alkylation[85] of the enolate dianion of **91** with 3-butenol triflate gave



Scheme 13. Synthesis of oscillarin by Hanessian et al. Azonia-Prins halocarbocyclization route to L-Choi derivative **98**. a) LHMDS, THF, −78 °C, then 3-butenol triflate; b) TFA, CH_2Cl_2; c) Toluene, reflux; d) Boc_2O, NEt_3, DMAP, CH_2Cl_2; e) $LiHBEt_3$, THF, −78 °C; f) Ac_2O, NEt_3, DMAP, CH_2Cl_2; g) $SnBr_4$, CH_2Cl_2, −78 °C, 5 min; h) Bu_4NOAc, toluene, 50 °C; i) TFA, CH_2Cl_2.

intermediate **92** in high yield. Cyclization to **93** and conversion to the *O*-acetyl hemiaminal derivative **94** was achieved using the same procedure as that described for dysinosin A.[84] Upon treatment of **94** with $SnBr_4$ in CH_2Cl_2 at −78 °C, the brominated bicyclic product **97** was produced within 5 min at −78 °C. The fact that **97** was obtained in 78% yield as a single isomer is rationalized by an antiperiplanar approach of the alkene tether onto the iminium ion, as in transition-state model **96**. The alternative conformer **95** is expected to be disfavored owing to the less favorable orbital overlap associated with a synclinal approach, as well as a possible steric clash between the ester substituent and the axial H-4. Quenching of the incipient carbocation by bromide in an equatorial fashion then gives **97**. This hypothesis is supported by a recent study of the steric and stereoelectronic effects of various nucleophilic tethers on related azonia-Prins halocarbocyclizations.[91] Elaboration of **97** to appropriately protected L-Choi derivative **98** was achieved by displacement of the bromide with tetrabutylammonium acetate and removal of the Boc group. Coupling of Choi amine **98** to fragment **99** proceeded in high yield and was followed by cleavage of the acetate and MOM protection to give **100** (Scheme 14). Finally, ester hydrolysis and condensation with the Adc subunit gave fully protected oscillarin, which, upon treatment with aqueous HCl in THF and purification by RP-HPLC gave the pure natural product **20** as the HCl salt.



Scheme 14. Synthesis of oscillarin by Hanessian et al. Completion of the synthesis. a) **98**, EDC, HOBt, CH_2Cl_2; b) NaOMe, MeOH; c) MOMCl, DIEA, CH_2Cl_2; d) LiOH, THF/H_2O; e) **86**, EDC, HOBt, NEt_3, CH_2Cl_2; f) 6 N aq. HCl, THF, then RP-HPLC.

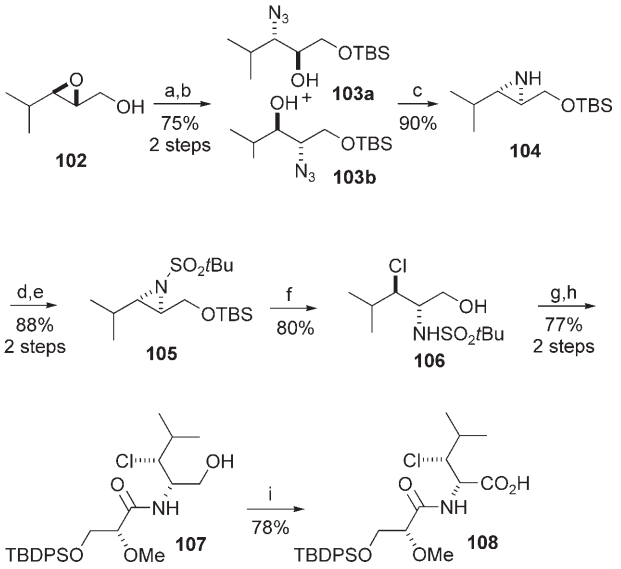
The spectral data obtained for **20** matched that reported for the natural product in every respect. As mentioned above, Hanessian and co-workers[43] had completed the synthesis of the orginially proposed structure of oscillarin (**19**), which featured a six-membered guanidine variant instead of the Adc subunit. The need for a structural revision became apparent upon comparison of the biological activities of **19** and **20**. While **20** proved to be a potent inhibitor of thrombin (IC_{50} = 28 nM, 0.018 μg mL^{-1}, Table 1), compound **19** showed no activity against the enzyme. The X-ray structure of the oscillarin–thrombin complex (Figure 4b) confirmed the structure of oscillarin and the Adc subunit.

4.6. Chlorodysinosin A

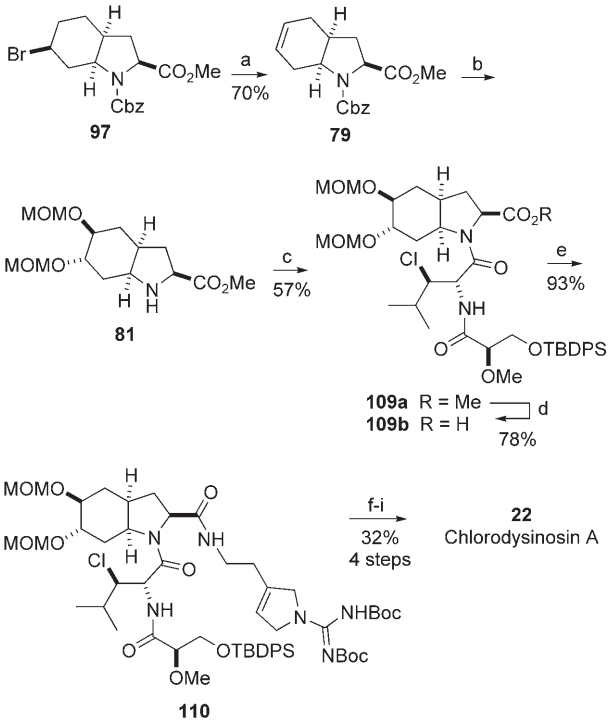
Most recently, Hanessian and co-workers reported the first total synthesis and structural confirmation of chlorodysinosin A (**22**),[46] which was originally disclosed in a patent by Pharmacia scientists in 2003[45] without any stereochemical assignment. Initial structural studies revealed that it differed from dysinosin A (**21**) only in the presence of a 3-chloroleucine amide subunit instead of D-leucine. Although the

stereochemistry of **22** was not assigned, its structural similarity to **21** suggested analogous absolute configurations. However, the configuration of the new stereocenter at C3 of the 3-chloroleucine subunit could be determined only through synthesis. The synthesis of the novel 3-chloroleucine-containing fragment (Scheme 15) commenced with the known epoxy alcohol **102**, obtained by Sharpless asymmetric epoxidation.[92] Aziridine **104** was obtained in three steps and activated as the *tert*-butylsulfonamide.[93] After a variety of conditions for regioselective cleavage of the aziridine had been screened, it was found that treatment of **105** with excess $CeCl_3 \cdot 7H_2O$ in refluxing acetonitrile provided the 3-chlorosulfonamide **106** with 10:1 regioselectivity. This reaction proceeded in 80% yield with concomitant cleavage of the silyl protecting group. The structure of the crystalline product was confirmed by X-ray diffraction. Interestingly, $CeCl_3$-mediated cleavage of the *N*-toluenesulfonyl- and *N*-trifluoromethylsulfonylaziridines proceeded with poor (<2:1) regioselectivity.[94] Bonjoch and co-workers[95] have recently described an alternative method to obtain diastereomeric 3-chloroleucines starting from β-lactones. Acidic deprotection of the *N*-Bus group and coupling with (*R*)-3-*tert*-butyldiphenylsilyloxy-2-methoxypropionic acid in the presence of PyBOP afforded **107** in 77% overall yield. Oxidation of **107** was achieved by employing H_5IO_6 and catalytic CrO_3,[96] after various other methods failed, in fact as a result of β elimination of the chloride.

Although the 5-hydroxy-L-Choi core of dysinosin A had been previously synthesized by an RCM aproach, Hanessian and co-workers exploited the intramolecular *N*-acyliminium ion halocarbocyclization reaction in a new synthesis of intermediate **79** (Scheme 16).[46] Thus, dehydrohalogenation of **97** by heating in neat DBU gave **79**, which was previously obtained by ring-closing metathesis of a bisalkene intermediate. Following the four-step protocol described for dysinosin A,[84] **79** was converted to **81** in preparation for coupling to the chloroacid fragment **108**.



Scheme 15. Synthesis of chlorodysinosin A by Hanessian et al. Synthesis of 3-chloroleucine fragment **108**. a) NaN_3, NH_4Cl, methoxyethanol, H_2O, reflux; b) TBSCl, NEt_3, DMAP, CH_2Cl_2; c) PPh_3, MeCN, 50°C; d) *t*BuSOCl, NEt_3, CH_2Cl_2; e) *m*CPBA, CH_2Cl_2; f) 4.0 equiv $CeCl_3 \cdot 7H_2O$, MeCN, 90°C, 72 h; g) 0.1 N TfOH in CH_2Cl_2, anisole; h) PyBOP, (*R*)-3-*tert*-butyldiphenylsilyloxy-2-methoxypropionic acid, 2,6-lutidine, CH_2Cl_2; i) 0.1 M H_5IO_6/wet MeCN, cat. CrO_3, 0°C.



Scheme 16. Synthesis of chlorodysinosin A by Hanessian et al. Completion of the synthesis. a) neat DBU, 80°C, 4 h; b) as in Scheme 10; c) **108**, DEPBT, 2,6-lutidine, CH_2Cl_2, 0°C, 4 h; d) 20 equiv MeSnOH, 1,2-DCE, 75°C, 48 h; e) **86**, PyBOP, 2,6-lutidine, CH_2Cl_2, 0°C, 4 h; f) 0.3 M TBAF/THF, 0°C, 15 min; g) SO_3·py, cat. Bu_2SnO, CH_2Cl_2, 18 h, then repeat; h) 10% TFA/CH_2Cl_2, RT, 6 h; i) preparative RP-HPLC.

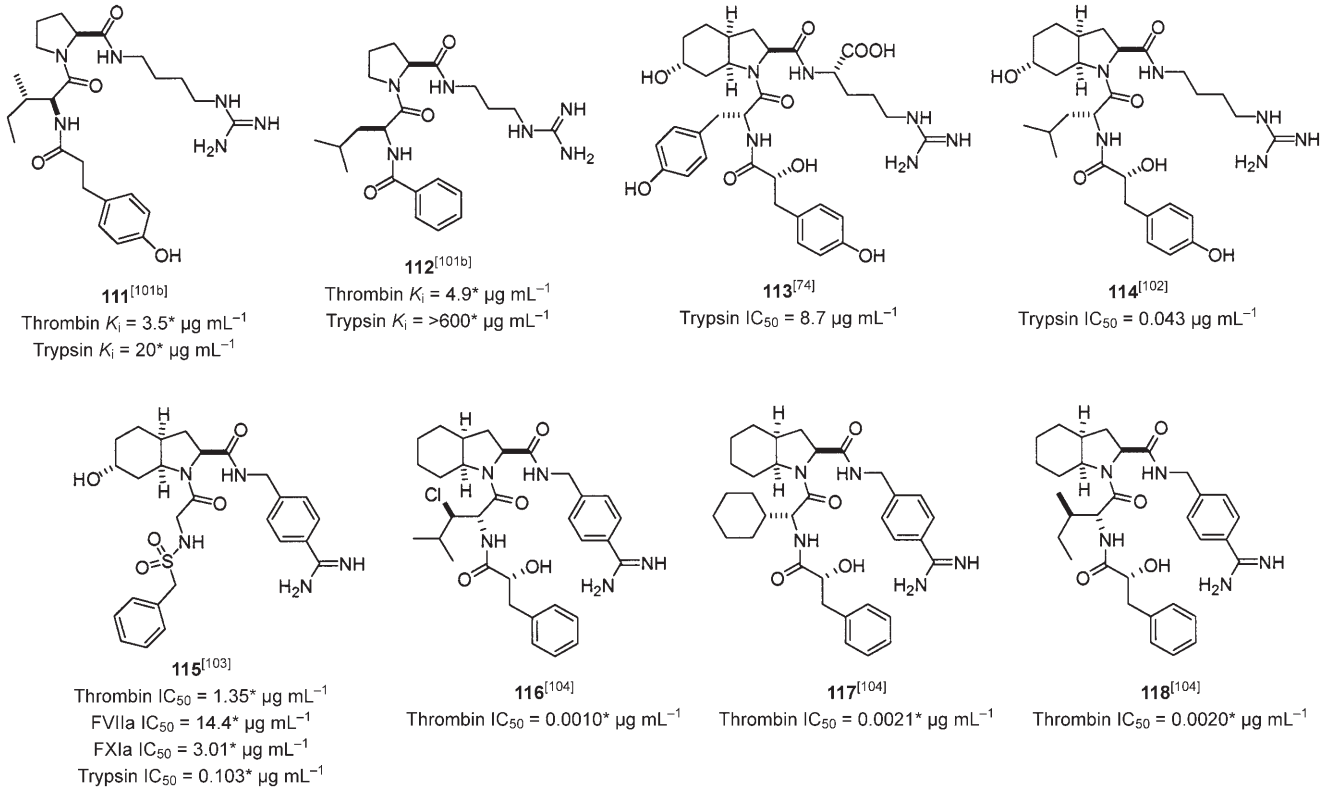
During assembly of chlorodysinosin A, it became evident that the base-sensitivity of the chlorine-containing intermediates would require a judicious choice of reagents and methods. In the condensation reaction of **108** and **81**, the use of triethylamine or diisopropylethylamine in combination with EDC/HOBt, HBTU/HOBt, or PyBOP resulted in significant elimination of HCl and low yields of coupled products. After extensive optimization, it was found that **109** could be obtained reproducibly in 55–60% yield in the presence of DEBPT[23,78,79] and 2,6-lutidine at 0°C. Hydrolysis of the methyl ester in **109a** also required the strict avoidance of alkaline conditions. By employing an excess of Me_3SnOH in refluxing 1,2-dichloroethane, smooth deesterification took place to give **109b** in 78% yield without dehydrochlorination.[97,98] Amide coupling to amine **86** in the presence of PyBOP then gave **110**.

Finally, cleavage of the silyl ether and sulfation[89,90] of the primary alcohol with SO_3·py and catalytic Bu_2SnO (twice) was followed by global deprotection with 10% TFA/CH_2Cl_2.

Pure chlorodysinosin A (**22**) was obtained in 32% yield from **110** after purification by RP-HPLC. The spectral data obtained from synthetic **22** matched that reported for the natural product in every respect, thus confirming the structure of chlorodysinosin A as well as the configuration of the novel (2*S*,3*R*)-3-chloroleucine residue. An X-ray crystal structure of the complex with thrombin provided conclusive evidence of the structural and configurational assignment through synthesis (Figure 4c).

5. Aeruginosin Analogues

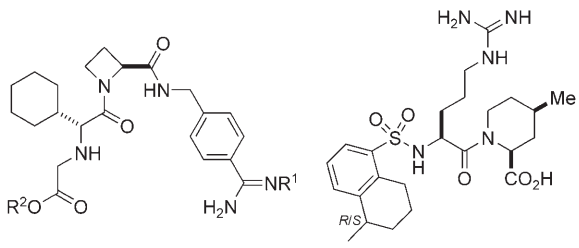
To date, limited efforts have been made to prepare analogues of the aeruginosin natural products.[74,99–104] Selected examples are depicted in Scheme 17. Mainly aeruginosins 298A and 98B have been explored as starting points for the synthesis of new synthetic aeruginosin-like serine protease inhibitors.[74,99–104] In one of these studies by Radau and co-workers[99] a trypsin activator rather than inhibitor was surprisingly identified. Radau and co-workers[99–101] used L-proline as a core structure instead of the synthetically more demanding Choi. More recently, Radau and co-workers[101b] have reported analogues possessing selective, albeit weak, inhibition against thrombin versus trypsin (**111** and **112**, Scheme 17). Shibasaki and co-workers,[74] and most recently Takahashi and co-workers[102] reported on the screening of aeruginosin 298A analogues against trypsin. Both of these studies supported the importance of the chemical structure of the basic P1 subunit for inhibitory activity. Manipulations in the P3 and P4 regions had a lower impact on the trypsin inhibition. Analogue **113** prepared by Shibasaki and co-workers[74] showed anti-trypsin activity comparable to that of the lead compound aeruginosin 298A. Furthermore, Takahashi and co-workers[102] found the potent inhibitor **114**, which is 300 times more active than aeruginosin 298A. Hanessian and co-workers[103] recently reported on truncated analogues of the aeruginosins with nonnatural benzamidine in the P1 position and including sulfamides, exemplified by compound **115** (Scheme 17). However, only modest activities against trypsin, thrombin, and other coagulation factors were obtained. A more thorough investigation of the SARs against thrombin was subsequently carried out by Hanessian and co-workers[104] focusing on the "chlorine effect" in chlorodysinosin A and probing the importance of all four aeruginosin subunits. The nonnatural benzamidine was found most favorable in the P1 position, while the size and shape of the P2 Choi core was proven less important. The beneficial "chlorine effect" in the P3 position was confirmed, and similar effects were obtained with other β-branched P3 side chains, such as the β-cyclohexyl (**117**) and isoleucine (**118**) analogues, which showed inhibitory activities against thrombin at $IC_{50} = 0.002\ \mu g\,mL^{-1}$. An N-terminal D-Pla was superior to simpler motifs, and the terminal sulfate group of chloriodysinosin A was shown to have only a small impact on the thrombin inhibition. To the best of our knowledge analogue **116**, lacking the 6-hydroxy group but with a benzamidine P1 unit, is the most active in vitro thrombin inhibitor reported to date ($IC_{50} = 0.0010\ \mu g\,mL^{-1}$, Scheme 17). Recently, Nie and co-workers[105] developed the synthesis of a ring-oxygenated variant of Choi starting from D-glucose, but no inhibitory activity has been reported.



Scheme 17. Examples of aeruginosin analogues. IC_{50} and K_i values reported as μM were converted to μg mL^{-1} and are indicated by an asterisk (*).

6. Clinical Application of Direct Thrombin Inhibitors

After a successful launching in 2004 of the thrombin inhibitor melagatran (**119**)[106] as the marketable, orally bioavailable prodrug ximelagatran (**120**, Exanta)[106] for



119[106], Melagatran: R^1=H, R^2=H
Thrombin K_i= 0.36 μM
120[106], Ximelagatran (Exanta): R^1=OH, R^2=Et
(prodrug)

121[108], Argatroban
Thrombin K_i= 0.039 μM

venous thrombosis, it had to be withdrawn in view of reported incidents of elevations in hepatic enzymes.[107] Argatroban (**121**), a 64:36 mixture of epimers at C21, lacks oral bioavailability and must be administered intravenously.[108] It is currently approved for treatment of thrombosis in patients affected by an immune-mediated response to heparin, which is the most widely used anticoagulant. According to recent reviews,[109] several synthetic thrombin inhibitors are in clinical trials at different stages.

7. Summary and Outlook

Natural products have, throughout history, been a valuable source of new molecular frameworks with diverse biological activities. The basic structural features of the aeruginosins appear to primarily incorporate the requisite pharmacophores for inhibition of trypsin-like serine proteases. Even though more than 20 natural members of the aeruginosin family have been isolated to date, more compounds are needed in order to more thoroughly map the SARs and identify important structural motifs for different biological activities. The development of efficient synthetic methodologies to the aeruginosins and their subunits will significantly facilitate the generation of new analogues. The identification of structural patterns for achieving selectivity between different human enzymes is also an important issue that requires further investigation.

From the result of the enzyme assays listed in Table 1 it is evident that different structural features are important depending on the target enzyme. A basic P1 end group appears to be a prerequisite for achieving high potency against the trypsin-like serine proteases. One explanation for the lack in activity against chymotrypsin and elastase might be the substrate preferences of these enzymes, which favor peptides with bulky hydrophobic or small neutral P1 side chains, respectively. Consequently, a replacement of the basic P1 subunit in the aeruginosins by a hydrophobic group might result in a shift in inhibitory activity towards other enzymes. The main role of the octahydroindole core subunit seems to be to direct different parts of the tetrapeptide into specific regions in space conferring the bioactive conformation. Fine-tuning of the octahydroindole substituents has been further suggested to attain selectivity between different enzymes.[26,36] Small changes in the nature of the P3 substituent on the amino acid residue appear to have a strong influence on the affinity for the coagulation factors. A striking example is the remarkable effect of the chlorine atom in chlorodysinosin A compared to the hydrogen-substituted dysinosin A (Table 1). The precise importance of the N-terminal sulfate group for different biological activities needs to be further elucidated.

Beside the search for better activities and selectivities against target serine proteases,[110] other structural and pharmacological aspects should be addressed to render this intriguing class of natural products more "druggable".[111] The prospects of achieving such a daunting task are nevertheless promising, especially since the natural products and the totally synthetic thrombin inhibitors share the same binding sites.[56–58,63,112–114] As such, they should be amenable to a segment-coupling protocol in a structure-based organic synthesis paradigm[115] to produce potent hybrid analogues with favorable pharmacological properties. It is hoped that future efforts in this area will lead to a safe and effective drug for the treatment of life-threatening thromboemboletic and related conditions.

Abbreviations

Adc	1-(*N*-amidino-Δ^3-pyrrolino)ethyl
Aeap	1-amidino-2-ethoxy-3-aminopiperidine
Agma	4-aminobutylguanidine
Alloc	allyloxycarbonyl
Argol	argininol
Argal	argininal
9-BBN	9-borabicyclo[3.3.1]nonane
BINOL	1,1′-bi-2-naphthol
Bn	benzyl
Boc	*tert*-butoxycarbonyl
BOP	benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate
BOPCl	bis(2-oxo-3-oxazolidinyl)phosphinic chloride
Bus	*tert*-butylsulfonyl
Bz	benzoyl
Cbz	benzyloxycarbonyl
Ccoi	2-carboxy-6-chlorooctahydroindole
Choi	2-carboxy-6-hydroxyoctahydroindole
Cleu	3-chloroleucine
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
1,2-DCE	1,2-dichloroethane
DEAD	diethyl azodicarboxylate
DEPBT	3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one
DEPC	diethyl pyrocarbonate
DIBAL-H	diisobutylaluminum hydride

DIEA	diisopropylethylamine
DMAP	4-*N*,*N*-dimethylaminopyridine
EDC	1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride
FDPP	pentafluorophenyl diphenyl phosphinate
HBTU	benzotriazol-1-yl-*N*-tetramethyluronium hexafluorophosphate
Hleu	3-hydroxyleucine
HOBt	1-hydroxybenzotriazole
Hpla	hydroxyphenyllactic acid
IBCF	isobutyl chloroformate
IC_{50}	inhibitor concentration resulting in 50% inhibition
ImH	imidazole
K_i	inhibition constant
LHMDS	lithium hexamethyldisilazide
L-Selectride	lithium tri-*sec*-butylborohydride
LS-Selectride	lithium trisiamylborohydride
*m*CPBA	*m*-chloroperoxybenzoic acid
MOM	methoxymethyl
MsCl	methanesulfonyl chloride
NMM	*N*-methylmorpholine
NOE	nuclear Overhauser effect
ODS HPLC	octadecylsilane high-pressure liquid chromatography
OH-Choi	2-carboxy-5,6-dihydroxyoctahydroindole
Pla	phenyllactic acid
Plas	phenyllactic acid-2-*O*-sulfate
PPACK	D-Phe-Pro-Arg-chloromethyl ketone
PyBOP	benzotriazol-1-yloxytri(pyrrolidino)phosphonium hexafluorophosphate
py	pyridine
RCM	ring-closing metathesis
SAR	structure–activity relationship
TBAF	tetrabutylammonium fluoride
TBDPS	*tert*-butyldiphenylsilyl
TBHP	*tert*-butyl hydroxyperoxide
TBS	*tert*-butyldimethylsilyl
TFA	trifluoroacetic acid
TfOH	trifluoromethanesulfonic acid
TIPS	triisopropylsilyl
TMSOTf	trimethylsilyl triflate

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