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Structure-based organic synthesis of unnatural aeruginosin hybrids as potent inhibitors of thrombin

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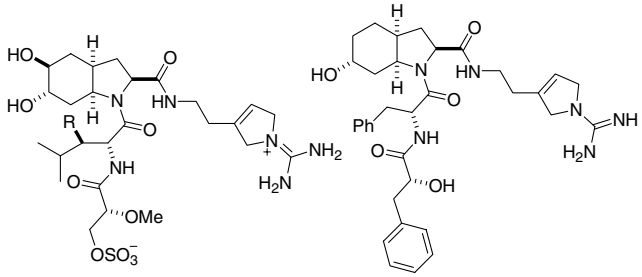
Abstract—Based on X-ray crystallographic data of complexes of chlorodysinosin A with the enzyme thrombin, a series of analogs were synthesized varying the nature of the P_1, P_2, and P_3 pharmacophoric sites and the central octahydroindole carboxyamide core. In general, introduction of a hydrophobic substituent on the D-leucine amide residue dramatically improved the inhibition of the enzyme. This is rationalized based on a better fit of the P_3 subunit in the hydrophobic S_3 enzyme site. Single digit nanomolar inhibition expressed as IC_{50} was observed for several analogs.

The 20-plus members of the aeruginosin family of potent serine protease inhibitors represent a relatively new class of so-called linear peptides encompassing a 6-mono- or 5,6-dihydroxy 2-octahydroindole carboxamide core unit (Choi and OHChoi, respectively).[1] They originate from geographically distinct aquatic sources that contain cyanobacterial organisms from which they can be isolated and characterized.[2] Reported outbreaks of livestock poisoning from contaminated water supplies harboring these cyan-colored algal blooms[3] attracted attention to the aeruginosins which were found to be non-toxic. Their structural novelty and interesting in vitro activity against serine proteases involved in the blood coagulation cascade leading to thrombus formation instigated structural studies with these enzymes, as well as efforts toward their total syntheses.[1] To date, seven members of the aeruginosin family have been synthesized, with four of these involving revisions to the originally proposed structures.[1] Potent in vitro activities of dysinosin A,[4] oscillarin,[5] and chlorodysinosin A[6] against thrombin and other factors in the intrinsic or extrinsic pathways to blood coagulation have been observed (Fig. 1).[7] Nearly all the aeruginosins contain a Choi or OHChoi central core unit upon which are appended pharmacophoric subunits that confer affinity to the catalytic binding pocket of trypsin, thrombin, and other serine proteases in this class (Fig. 2). Thus, a basic P_1 and hydrophobic P_2, P_3 main pharmaco-



Dysinosin A, R = H
Thrombin IC_{50} = 0.046 μM
FVIIa IC_{50} = 0.326 μM

Chlorodysinosin A, R= Cl
Thrombin IC_{50} = 0.0057 μM
FVIIa IC_{50} = 0.039 μM

Oscillarin
Thrombin IC_{50} = 0.028 μM
FVIIa IC_{50} = 3.9 μM

Figure 1. Structures and enzymatic activities of the natural aeruginosins chlorodysinosin A, dysinosin A, and oscillarin.

Keywords: Thrombin inhibition; Aeruginosin mimics; Chloroleucine.
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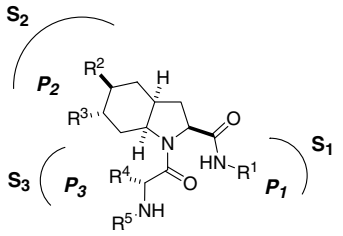
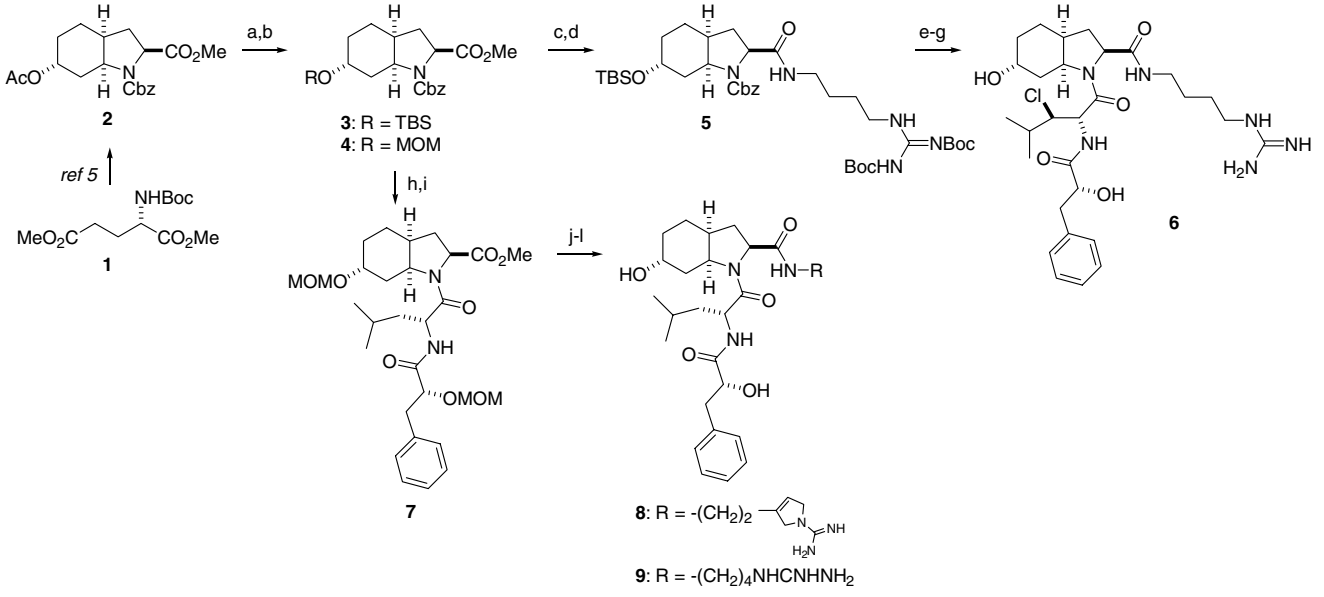


Figure 2. Binding mode of the aeruginosin family according to X-ray co-crystal structures complexed with thrombin.[2d,5,6,8]

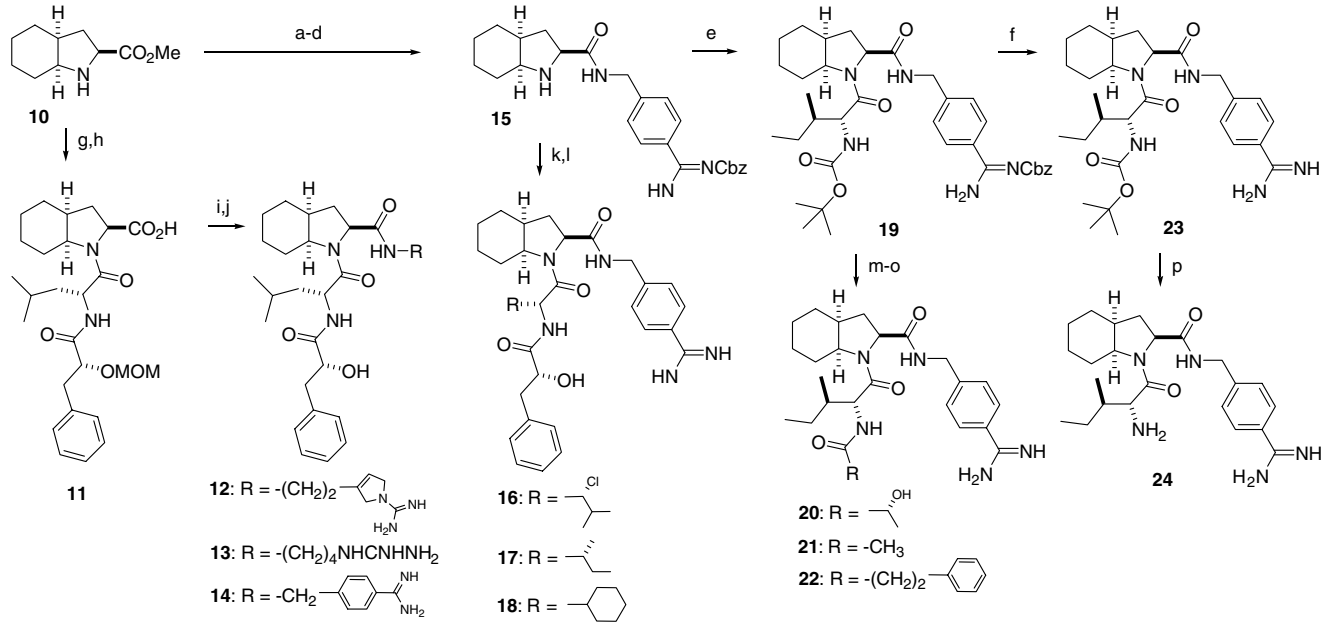
phoric subunits are strategically deployed for optimal binding.

The most recent entry in the family of aeruginosins, namely chlorodysinosin A, exhibited the highest in vitro inhibitory activity against thrombin (Factor IIA) and Factor VIIA (IC_{50} = 5.7 and 39 nM, respectively).[6] Even more remarkable was the dramatic effect of a chlorine atom present in the (2*S*,3*R*)-3-chloroleucine subunit, harboring the hydrophobic side-chain compared to dysinosin A which lacks the chlorine in the same amino acid unit (IC_{50} thrombin, 46 nM; Factor VIIA, 326 nM).[4] X-ray co-crystal structure data of chlorodysinosin A (2GDE)[6] (and dysinosin A),[2d] as well as molecular modeling[6], delineated the possible reasons for this remarkable 'chlorine effect' especially that the inhibitors (and amino acid residues in the enzyme) did not undergo major positional or conformational changes. We have ascribed this unexpected 'chlorine effect' to a more restricted χ^1 angle, possibly positioning the chlorine atom in a spacially favorable orientation in the S_3 pocket with a better entropic gain and associated with loss of water molecules.[6] To validate this effect, we embarked on the synthesis of hybrid aeruginosins by segment coupling of natural and unnatural pharmacophoric units representing the P_1 and P_3 units anchored upon the perhydroindole carboxylic acid core. Variations in the P_1 subunit consisted of the 'natural' 1-amidino-Δ^3 pyrroline and agmatine groups, and the unnatural 4-amidinobenzylamino group.[9] The P_3 subunit was varied to include D-leucine, D-3*R*-chloroleucine, D-isoleucine, and D-cyclohexyl glycine. The perhydroindole carboxylic acid core consisted of 5-mono- and 5,6-dihydroxy analogs, as well as the unsubstituted 'unnatural' variant. Finally, the 'acidic' appendage was patterned after oscillarin[5] containing a D-phenyllactic acid end-group, in addition to simpler motifs. This provided a representative set of natural and unnatural hybrid aeruginosin-like molecules to probe the importance of each unit and functional group in conferring inhibitory activity against thrombin as a principal enzyme. Most anticipated was to validate the beneficial 'chlorine effect' in selected analogs.

The Choi core structure **2** was prepared from the protected L-glutamic acid **1** via dianion alkylation and azonia-Prins halocarbocyclization, as previously described (Scheme 1).[5,10] The corresponding unsubstituted core structure **10** in Scheme 2 was obtained quantitatively from commercially available L-Oic (2-octahydroindole carboxylic acid) using TMSCl in MeOH. In order to install the diversity in the last step of the syntheses, different routes were taken to the final aeruginosin hybrids **6**, **8**, **9**, **12**–**14**, **16**–**18**, and **20**–**24** as shown in Schemes 1 and 2. Depending on which side-chain was varied, P_1 or P_3, the C- and N-terminal segments were coupled to the azabicyclic core structures in different orders. Standard peptide coupling conditions were used utilizing PyBOP



Scheme 1. Synthesis of aeruginosin analogs comprising the L-Choi subunit (**6**, **8**, and **9**). Reagents and conditions: (a) NaOMe, MeOH; (b) MOMCl, DIEA, CH_2Cl_2 (99%, 2 steps) or TBSOTf, NEt_3, CH_2Cl_2 (85%, 2 steps); (c) LiOH, H_2O/THF, quant; (d) N^2,N^3-diBoc-agmatine,[12] PyBOP, 2,6-lutidine, CH_2Cl_2, 84%; (e) H_2, $Pd(OH)_2$/C, EtOAc, 95%; (f) **29**, DEPBT, 2,6-lutidine, CH_2Cl_2, 37%; (g) wet TFA/CH_2Cl_2 3:7–1:1, 61%; (h) H_2, Pd/C, MeOH, 95%; (i) **26**, DEPBT, 2,6-lutidine, CH_2Cl_2, 84%; (j) LiOH, H_2O/THF, 95%; (k) 2-(*N*,*N*′-diBoc-*N*-amidino-Δ^3-pyrrolino)ethylamine[4] or N^2,N^3-diBoc-agmatine,[12] PyBOP, 2,6-lutidine, CH_2Cl_2; (l) concd HCl (aq)/THF 1:1 (**8**, 37%, 2 steps) or wet TFA/CH_2Cl_2 9:1 (**9**, 51%, 2 steps).

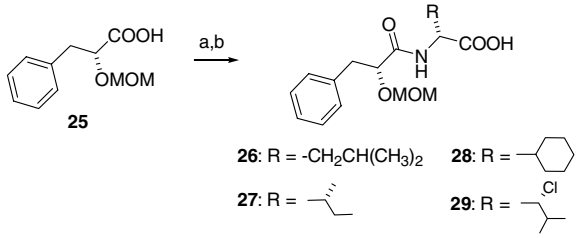


Scheme 2. Synthesis of aeruginosin analogs comprising the L-Oic subunit (**12**–**14**, **16**–**18**, and **20**–**24**). Reagents and conditions: (a) Boc_2O, NEt_3, MeOH, 96%; (b) LiOH, H_2O/THF, 86%; (c) *N*-Cbz-4-amino-methylbenzamidine, PyBOP, 2,6-lutidine, DMF, 89%; (d) TFA/CH_2Cl_2 1:9, 99%; (e) *N*-Boc-D-isoleucine, DEPBT, 2,6-lutidine, CH_2Cl_2, 62%; (f) H_2, Pd/C, MeOH, 73%; (g) **26**, DEPBT, 2,6-lutidine, CH_2Cl_2, 71%; (h) LiOH, H_2O/THF, 98%; (i) 2-(*N*,*N*′-diBoc-*N*-amidino-Δ^3-pyrrolino)ethylamine,[4] *N*-Cbz -4-amino-methylbenzamidine or N^2,N^3-diBoc-agmatine,[12] PyBOP, 2,6-lutidine, CH_2Cl_2 or DMF; (j) concd HCl (aq)/THF 1:1 (**12**, 46%, 2 steps, **13**, 72%, 2 steps) or H_2, Pd/C, MeOH then concd HCl (aq)/THF 1:1 (**14**, 61%, 3 steps); (k) **27**, **28**, or **29**, DEPBT, 2,6-lutidine, CH_2Cl_2; (l) H_2, Pd/C, MeOH, then wet TFA/CH_2Cl_2 9:1 (**16**, 30%, 3 steps, **17**, 35%, 3 steps, and **18**, 65%, 3 steps); (m) TFA/CH_2Cl_2 1:9, 91%; (n) D-lactic acid, PyBOP, 2,6-lutidine, CH_2Cl_2/DMF, or acetylchloride or hydrocinnamoylchloride, NEt_3, CH_2Cl_2; (o) H_2, Pd/C, MeOH (**20**, 77%, **21**, 70%, **22**, 53%, over 2 steps); (p) TFA/CH_2Cl_2 1:9, 95%.

or DEPBT as coupling reagents and 2,6-lutidine as base. PyBOP was used in the couplings of the basic C-terminal P_1 groups, while DEPBT was employed to install the N-terminal segments (Schemes 1 and 2). DEPBT was previously successfully employed in the amide coupling between the OHChoi nitrogen and the N-terminal segment in the synthesis of the 'natural' chlorodysinosin A.[6] The N-terminal end groups in hybrids **20**–**22** were incorporated in the last step before deprotection, either via PyBOP coupling with D-lactic acid to get **20**, or via direct coupling with the acid chlorides to obtain **21** and **22** (Scheme 2). Finally, global deprotection using acidic conditions or hydrogenation followed by treatment with acid afforded the target hybrids **6**, **8**, **9**, **12**–**14**, **16**–**18**, and **20**–**24**. Treatment with TFA in dichloromethane resulted in a cleaner reaction compared to concd HCl in THF. However, the Choi- and OHChoi-containing hybrids had to be treated with 10% aq NaOH prior to purification on RP-HPLC in order to saponify any trifluoroacetylated Choi-hydroxyls.[11]

The N-terminal segments **26**–**28** were prepared from the MOM-protected D-phenyllactic acid **25** essentially as previously reported (Scheme 3).[5] The route to the 3-chloroleucine N-terminal segments **29** was prepared similarly as in the synthesis of chlorodysinosin A,[6] with oxidation to the carboxylic acid in the last step using H_5IO_6 and cat. CrO_3[13] (Scheme 3).

In an effort to investigate the impact of the acidic sulfate group in chlorodysinosin A the non-sulfated **31** was pre-

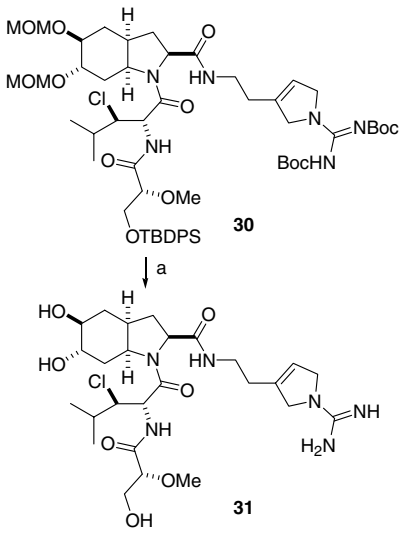


Scheme 3. Synthesis of the N-terminal segments **26**–**29**. Reagents and conditions: (a) *O*-Bn-D-Leu, *O*-Me-D-Ile or *O*-Me-D-Chg, EDC, HOBt, NEt_3, CH_2Cl_2 or CH_2Cl_2/DMF or (2*S*,3*R*)-3-chloroleucinol, PyBOP, 2,6-lutidine, CH_2Cl_2; (b) H_2, Pd/C, MeOH (**26**, 94%, 2 steps) or LiOH, H_2O/THF (**27**, 82%, 2 steps, **28**, 80%, 2 steps) or 0.4 M H_5IO_6/wet MeCN, cat. CrO_3, 0 °C (**29**, 51%, 2 steps).

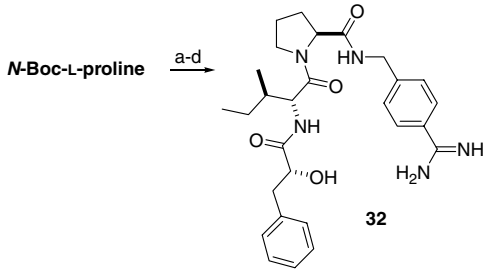
pared by deprotection of the precursor **30** using TFA in dichloromethane (Scheme 4).

A proline-containing hybrid **32** analogous to the Oic-hybrid **17** was synthesized as a reference compound to fully investigate the effect of the size of the P_2 subunit. The same synthetic route as to hybrid **17** was employed (Scheme 5).

The aeruginosin hybrids **6**, **8**, **9**, **12**–**14**, **16**–**18**, **20**–**24**, **31**, and **32** were evaluated in enzymatic assays for their inhibitory activity against thrombin. The results are presented as IC_{50} values in Table 1.[14] Activities ranging from low nanomolar to micromolar IC_{50} values were obtained depending on the specific combination of P_1–P_3 pharmacophoric subunits.[15] In accordance with



Scheme 4. Synthesis of non-sulfated chlorodysinosin A **31**. Reagents: (a) wet TFA/CH_2Cl_2 9:1, 95%.



Scheme 5. Synthesis of L-proline-containing aeruginosin analog **32**. Reagents and conditions: (a) *N*-Cbz-4-amino-methylbenzamidine, PyBOP, 2,6-lutidine, DMF, 93%; (b) TFA/CH_2Cl_2 1:9, quant.; (c) **27**, DEPBT, 2,6-lutidine, CH_2Cl_2/DMF, 70%; (d) H_2, Pd/C, MeOH, then wet TFA/CH_2Cl_2 9:1 (82%, 2 steps).

previous observations,[1] the shape and orientation of the basic P_1 side-chain was found to be of vital importance for high inhibitory activity. In general, the 4-amidinobenzyl group was preferred in the P_1 position over agmatine and 1-amidino-Δ^3 pyrroline (cf. **12**, **13**, and **14**, Table 1).

The azabicyclic P_2 subunit has mainly been suggested as a device conferring the bioactive conformation in the aeruginosin-like molecules. Apparently, the 6-hydroxyl of Choi makes no difference for the inhibitory activity when comparing to the unsubstituted Oic-hybrids (cf. **8** vs **12** and **13** vs **9**, Table 1). In fact even slightly improved activities were obtained without the 6-hydroxyl against thrombin. The activity of the proline hybrid **32** was noteworthy.[16]

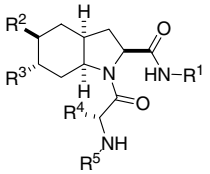
Encouraged by the remarkable effect of a 3*R*-chloro substituent in the P_3 side-chain (cf. **6** vs **9**, Table 1) a series of β-branched hybrids **16**, **17**, and **18** incorporating the most potent 4-amidinobenzyl P_1 group and the unsubstituted Oic P_2 subunit were prepared and evaluated. To the best of our knowledge the D-3*R*-chloroleucine hybrid **16** identified in this small series is the most active thrombin inhibitor of the natural aeruginosins and the aeruginosin-derived analogs reported to date (IC_{50} = 0.0016 μM, Table 1). Hybrids **17** and **18** encompassing a D-isoleucine or D-cyclohexyl glycine in the P_3 position demonstrated activities against thrombin in the same low nanomolar range confirming a strong preference for β-branched P_3 side-chains. Molecular dynamics simulations indicate that this so-called 'chlorine effect' primarily originates from a stabilization by the β-substituent of the χ^1 angle in the bioactive conformation rather than from a general hydrophobic effect, which is also reflected by the similar *c*log *P* values of the hybrids with and without a β-chloro substituent (cf. **9**, *c*log *P* = 0.296 with **6**, *c*log *P* = 0.359 and **14**, *c*log *P* = 3.91 with **16**, *c*log *P* = 3.97).[17] Moreover, the bioactive conformation places the β-substituent in a favorable position in the S_3 subsite resulting in a release of water and thus a gain in entropy as well as additional hydrophobic interactions with the enzyme pocket. According to the contour diagram in Figure 3 there seems to be enough space in the S_3 subsite to accommodate even larger β-substituents in aeruginosin hybrids. Futhermore, D-phenylalanine appears to be preferred over D-leucine in the P_3 position. Compound **8**, which is the P_3 D-leucine hybrid of the natural oscillarin, was less potent than the D-phenylalanine-containing natural product (IC_{50} = 0.22 and 0.028 μM against thrombin, respectively).

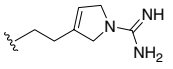
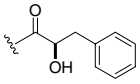
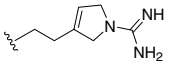
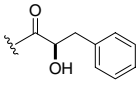
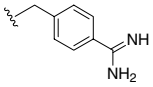
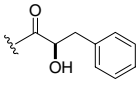
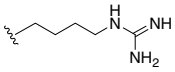
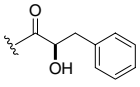
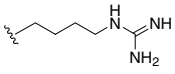
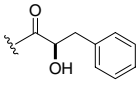
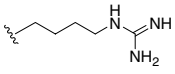
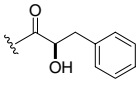
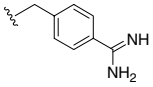
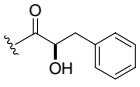
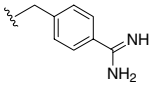
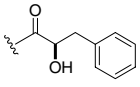
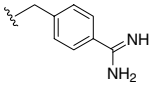
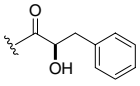
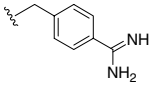
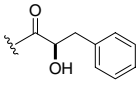
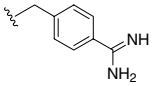
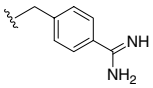
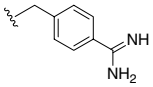
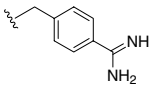
The oscillarin D-phenyllactic acid was chosen as a general N-terminal appendage in this series of hybrids. Oscillarin, which is lacking an N-terminal acidic group, is one of the most active thrombin inhibitors of the natural aeruginosins (Fig. 1).[5] The avoidance of an acidic group was considered favorable both from synthetic and ADME perspectives. It was also found that the gain in activity due to an N-terminal acidic group appears to be very modest. A comparison between the non-sulfated chlorodysinosin A **31** and chlorodysinosin A shows only a slight increase in activity against thrombin with a sulfate end group (IC_{50} = 0.0057 and 0.011 μM, respectively). D-Phenyllactic acid was found to be superior to other simpler N-terminal end groups (cf. compound **17** with **20**–**24**, Table 1). The loss in inhibitory activity was more pronounced when omitting the hydroxyl than the terminal phenyl group, cf. **22** and **20**. A complete truncation of the N-terminal appendage resulted in amine **24**, which still demonstrated significant activity against thrombin (IC_{50} = 0.101 μM, Table 1).

The comparatively stronger inhibition of compound **6** than its des-chloro analog **9** suggests that the structure of aeruginosin 205B should be revised, with the chloro atom on the D-leucine residue, rather than on Choi.[18]

In conclusion, a series of unnatural aeruginosin hybrids have been synthesized to map the structure–activity relationships of the P_1, P_2, P_3, and the N-terminal subunits against thrombin. A pronounced increase in activity was found when incorporating a β-substituent on the P_3 side-chain, establishing the previously acknowledged 'chlorine effect' of the D-3*R*-chloroleucine in chlorodysinosin A.

Table 1. Inhibition of thrombin measured as IC_{50} values



Compound	R^1	R^2	R^3	R^4	R^5	IC_{50} (μM) Thrombin
8		H	OH			0.22
12		H	H			0.12
14		H	H			0.097
13		H	H			1.5
9		H	OH			5.6
6		H	OH			0.31
16		H	H			0.0016
17		H	H			0.0033
32 L-proline		-(L-proline)	-(L-proline)			0.007
18		H	H			0.0034
24		H	H		H	0.101
21		H	H			0.786
20		H	H			0.053
22		H	H			0.334
23		H	H			0.171
31		OH	OH			0.011

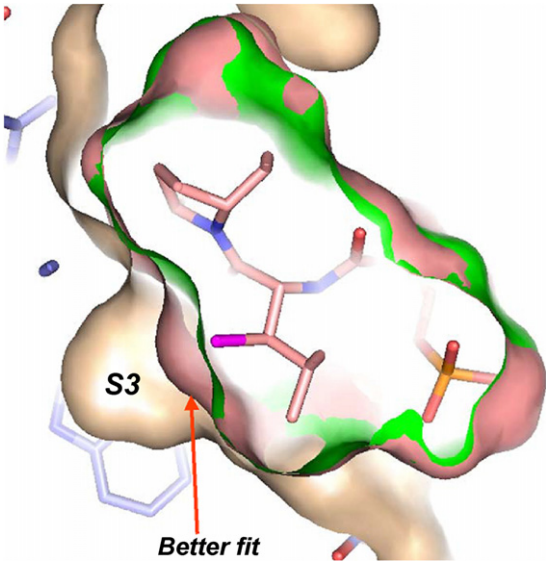


Figure 3. Contour diagram of the S_3 pocket and the P_3 side-chains from an overlay of the co-crystal structures of dysinosin A[2d] (green) and chlorodysinosin A[6] (2GDE, pink) with thrombin.

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