Pharmazeutische/Medizinische Chemie[1], Institut für Pharmazie der Ernst-Moritz-Arndt-Universität Greifswald, Zentrum für Vaskuläre Biologie und Medizin[2], Klinikum der Friedrich-Schiller-Universität Jena, Erfurt, Germany

Cyanopeptide analogues: new lead structures for the design and synthesis of new thrombin inhibitors

G. RADAU[1] and J. STÜRZEBECHER[2]

This contribution deals with the structure-based design and syntheses of the new serine protease inhibitors **RA-1001** and **RA-1002**, which are analogues of the blue-green algae derived cyanopeptide aeruginosin 98-B. Both compounds inhibit thrombin with K_i values of 5.6 μM and 8.7 μM, respectively.

1. Introduction

Recently, blue-green algae (cyanobacteria) have become targets for screening programmes in search of novel compounds of potential medicinal value. Cyanopeptides, the peptidic metabolites of blue-green algae, possess a broad spectrum of biological activities including inhibition of serine proteases [1], antitumor [2], immunosuppressive [3], and antimicrobial [4] effects as well as angiotensin-converting enzyme inhibitory action [5] and cardioactive effects [6]. In the following we report on the first study of designing serine protease inhibitors using cyanopeptides as new lead structures.

Trypsin-like serine proteases play central roles in human organism. The most crucial member of this enzyme family, thrombin, controls the balance between hemostasis and fibrinolysis. Thrombin converts the soluble plasma protein, fibrinogen, into the insoluble protein fibrin, which forms the matrix of blood clots. Thrombin also activates factor XIII (fXIII), which catalyses covalent cross-linking of fibrin (stabilisation of the clot). It promotes and amplifies clot formation by activating other coagulation factors including fV and fVIII. Thrombin stimulates platelet aggregation through proteolytic cleavage of the thrombin receptor. In addition to prothrombotic functions, thrombin mediates an antithrombotic effect by activating protein C after binding to the cell surface protein, thrombomodulin.

Defects in the various pathways leading to the generation of thrombin are associated with bleeding diatheses or thrombotic complications, including venous and arterial thrombosis, stroke, restenosis, and recurrent myocardial infarction. The discovery of oral thrombin inhibitors therefore presents a notable measure for improving the treatment of the above mentioned disorders.

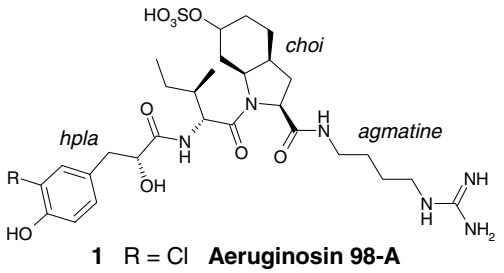
2. Investigations, results, and discussion

In search of new lead structures for designing thrombin inhibitors we focused our attention on linear cyanopeptides, in particular on the family of the aeruginosins as typical representatives.

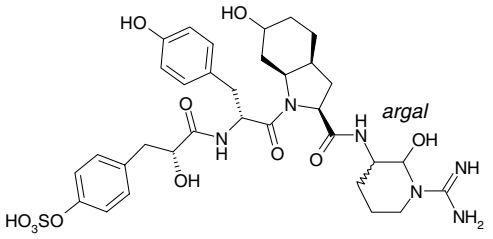
2.1. Structural considerations

Aeruginosins **1**–**5** contain the unusual 2-carboxy-6-hydroxy-octahydroindole (choi) as a common amino acid, the acidic 4-hydroxyphenyllactic acid (hpla) side-chain, and finally the basic arginine-derived side-chaines. Amino acids in P3 are represented by D-allo-Ile, L-Phe, and L-Leu.

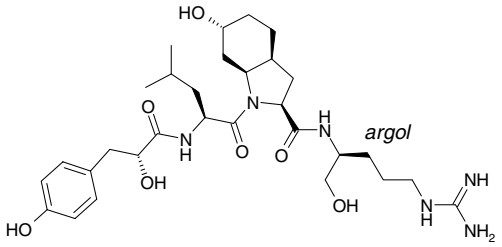
Aeruginosins 98-A and B (**1** and **2**), isolated from *Microcystis aeruginosa* [7], are equipotent inhibitors of trypsin, plasmin, and thrombin (IC_{50} values see Table 1). In compounds **1** and **2**, isoleucine shows D-allo configuration; if



1 R = Cl **Aeruginosin 98-A**
2 R = H **Aeruginosin 98-B**



3 L-Argal **Aeruginosin 102-A**
4 D-Argal **Aeruginosin 102-B**



5 **Aeruginosin 298-A**

Table 1: Inhibititory data of aeruginosins 1–5

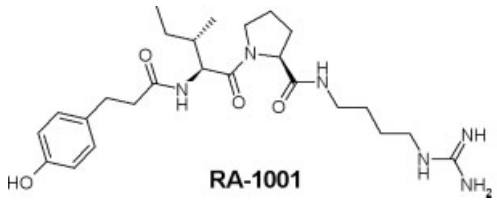
Compd.	IC_{50} (μg/ml)		
	Trypsin	Thrombin	Plasmin
1	0.6	7.0	6.0
2	0.6	10.0	7.0
3	0.2	0.04	0.3
4	1.1	0.1	0.8
5	1.0	0.3	–

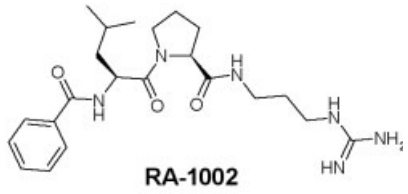
the residue was a L- or L-allo-isoleucine, the side-chain would produce steric conflicts with the bulky choi sulfate. Agmatine constitutes the basic side-chain and is essential for inhibiting trypsin-like serine proteases. The absolute configuration of **2** was determined by X-ray crystallographic studies of the cocrystallized **2**-trypsin-complex (pdb entry: 1aq7) [8]. Unique is the conspicuous lack of any interactions of **2** with trypsin's catalytic triad (Ser195, His57, Asp102). The Ser195 residue does not show close contacts to any atoms of **2**; this may indicate that a new mode of inhibition differing from the standard mechanisms of serine protease inhibitors is possible.

Aeruginosins 102-A and B (**3** and **4**, *Microcystis viridis*) inhibit thrombin and plasmin more effective than **1** and **2**. Compound **3** inhibits thrombin stronger than **4**; the different stereocenters of argal appear to be the reason for this different behaviour [9].

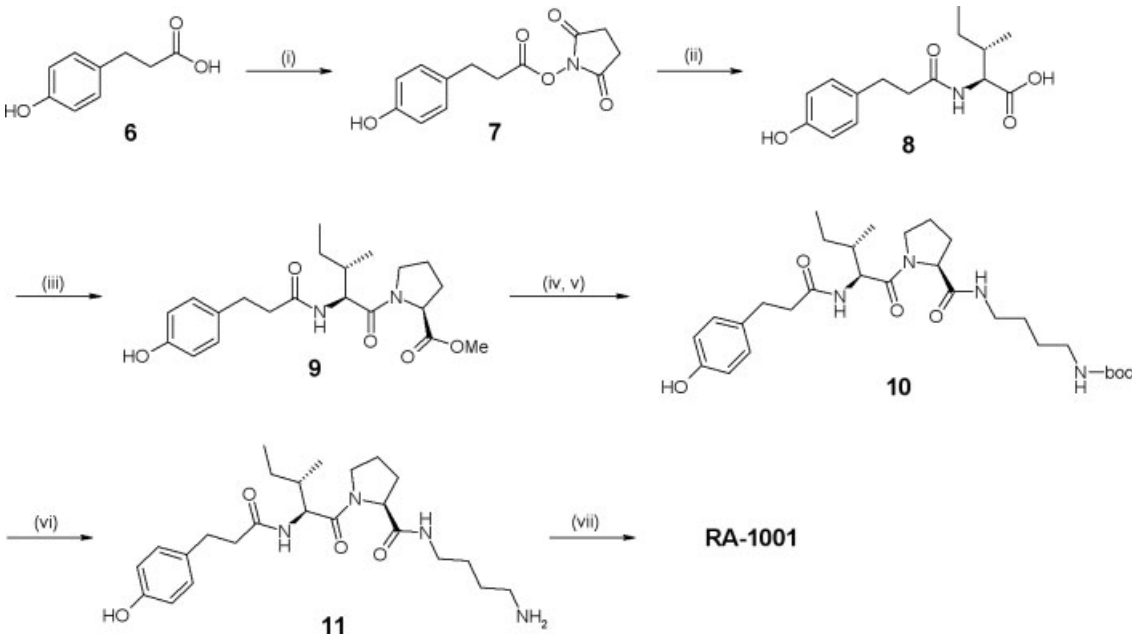
Aeruginosin 298-A (**5**, *M. aeruginosa*) has a more favourable structure for the inhibition of trypsin and thrombin, with IC_{50} values comparable to aeruginosin 102-B (**4**). Argol, the basic side-chain of **5**, is more flexible than argal. For this reason, **5** fits better into the hydrophobic pocket [10]. X-ray data of the cocrystallized **5**-thrombin-complex confirm that the hydroxy group of argol forms a hydrogen bond with His57, which destroys the original hydrogen bonding system of the catalytic triad. The choi unit binds to the S2-binding-subsite in the usual way. Even though leucine is a L-amino acid, it partially occupies the hydrophobic, D-enantiomorphic S3-binding subsite [11].

Because of its structural features, we decided to choose aeruginosin 98-B (**2**) as a lead structure for the design of new serine protease inhibitors. Although **3** inhibits thrombin better than **2**, the synthesis of the argal subunit seemed to be more difficult and time-consuming than the synthesis of agmatine. Finally, the unusual mode of inhibition – there are no interactions between **2** and the catalytic triad of trypsin – encouraged us to synthesise the analogues **RA-1001** and **RA-1002**. Since X-ray data of the **2**-trypsin-complex suggest that the six-membered ring of choi protrudes into the solvent [8], this ring seemed not to be necessary to inhibit trypsin or thrombin. Thus, choi was replaced with L-proline; this modification enables the connection of proline with L-isoleucine without fearing any steric conflicts similar to those between L-isoleucine and choi. Furthermore, hpla was replaced by 3-(4-hydro-



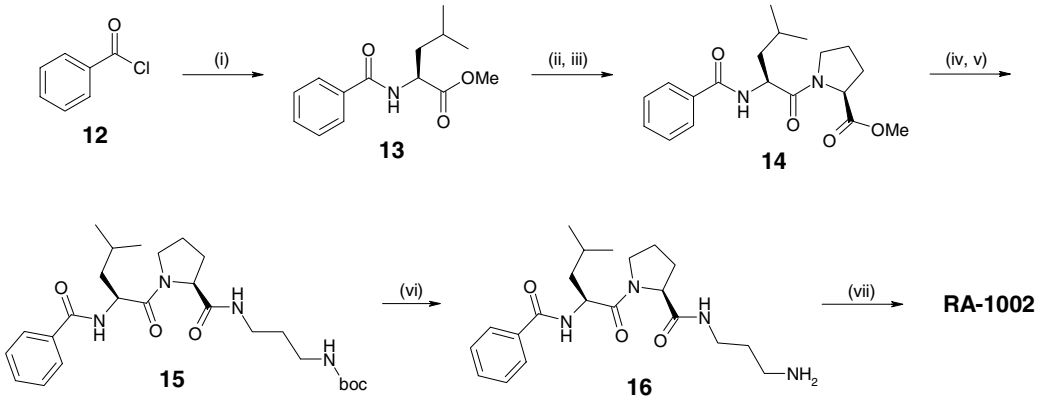


Scheme 1



i: DCC, NHS, EA, rt, 78%; ii: $NaHCO_3$, L-Ile, EtOH, H_2O, rt, 76%; iii: DCC, EA, L-Pro-OMe, DIPEA, 0°C, 84%; iv: LiOH (1M), DME, rt, quant.; v: 1-amino-4-(tert.-butyloxycarbonyl)-aminobutane, DIPCI, DCM, 0°C, 26%; vi: TFA, DCM, 0°C, 66%; vii: S-methylisothiouronium iodide, DIPEA, H_2O, 94% [12].

Scheme 2



i: DIPEA, EA, DCM, L-Leu-OMe, rt, 61%; ii: LiOH (1M), DME, rt, 98%; iii: DCC, EA, DCM, L-Pro-OMe, DIPEA, 0°C, 52%; iv: LiOH (1M), DME, rt, 81%; v: 1-amino-3-(tert.-butyloxycarbonyl)aminopropane, DIPCI, DCM, 0°C, 60%; vi: TFA, DCM, 0°C, 49%; vii: S-methylisothiouronium iodide, DIPEA, H_2O, 52%.

xyphenyl)propionyl and benzoyl moieties, respectively. Finally, the length of the basic side-chain was shortened by one carbon atom.

2.2. Syntheses

At the beginning of the synthesis of **RA-1001**, the succinimide ester of 3-(4-hydroxyphenyl)propionic acid (**7**) reacted with completely unprotected L-isoleucine to give the acylated amino acid (Scheme 1). Activation of **8** by DCC and reaction of the *in situ* generated active ester with L-proline methyl ester hydrochloride gave the appropriate dipeptide methyl ester **9**. Hydrolysis of the ester and activation by DIPCI enabled L-proline to react with mono-BOC-substituted 1,4-diaminobutane. Cleavage of the protection group by means of a TFA/DCM mixture (1 : 1) furnished the primary amine **11**, which was converted into **RA-1001** through substitution with *S*-methylisothiouronium iodide.

The synthesis of **RA-1002** started with benzoylation of L-leucine methyl ester hydrochloride (Scheme 2). *N*-Benzoyl-isoleucine methyl ester (**13**) was saponified and the resulting acid was transformed into the dipeptide methyl ester **14** by condensation with L-proline methyl ester. Repeated hydrolysis, activation, and reaction with mono-BOC-substituted diaminopropane yielded the appropriate derivative **15**. Compound **RA-1002** was synthesised by cleavage of the BOC protecting group and subsequent reaction of the resulting TFA salt with S-methylisothiouronium iodide under basic conditions (DIPEA).

2.3. Inhibition tests

As expected, the guanidine derivatives **RA-1001** and **RA-1002** are potent thrombin inhibitors with K_i values of 5.6 μM and 8.7 μM, respectively. **RA-1001** is the only member of the synthetic aeruginosin analogues shown in Table 2 that is able to inhibit trypsin. In contrast to **RA-1001**, aeruginosin 98-B (**2**) inhibits trypsin stronger than thrombin. The reason for this different behaviour may be caused by steric conflicts between aeruginosin's bulky choi group and the S2 pocket of thrombin, which are less prominent in the proline moiety of **RA-1001**. Surprisingly, the synthetic precursor of **RA-1001**, primary amine **11**, inhibits thrombin in the same order of magnitude. The precursor of **RA-1002**, amine **16**, shows a K_i value of 62 μM for the inhibition of thrombin. None of the four analogues decreases the activity of the trypsin-like serine proteases factor Xa and tryptase.

Table 2: Inhibitory data of synthetic aeruginosin analogues

Compd.	K_i (μM)			
	Trypsin	Thrombin	f. Xa	Tryptase
11	>1000	9.0	>1000	>1000
16	>1000	62	>1000	>1000
RA-1001	32	5.6	>1000	>1000
RA-1002	>1000	8.7	>1000	>1000

In summary, we have developed three equipotent thrombin inhibitors (**11**, **RA-1001**, and **RA-1002**) based on the structure of the cyanopeptide aeruginosin 98-B. Moreover, compounds **11**, **16**, and **RA-1002** seem to be selective inhibitors of thrombin. Further SAR studies (especially in P3 and P4 moieties) and X-ray crystal structures – first X-ray studies of the **RA-1001**-trypsin-complex confirm an aeruginosin-analogous binding mode – are currently in process to increase the inhibitory potency and selectivity of both new lead structures **RA-1001** and **RA-1002**.

3. Experimental

3.1. Abbreviations

BOC: *tert.*-butyloxycarbonyl, DCC: *N,N′*-dicyclohexylcarbodiimide, DCM: dichloromethane, DIPCI: *N,N′*-diiso-propylcarbodiimide, DIPEA: diiso-propylethylamine, DME: 1,2-dimethoxyethane, EA: ethyl acetate, NHS: *N*-hydroxysuccinimide, PE: petroleum ether, rt: room temperature, TFA: trifluoroacetic acid.

3.2. Typical synthesis procedures

*3.2.1. Synthesis of **7***

To an ice-cooled solution of 3-(4-hydroxyphenyl)propionic acid (10.0 mmol) and NHS (10.0 mmol) in EA (30 ml) a solution of DCC (10.0 mmol) in EA (15 ml) was added dropwise over a period of 15 min. While stirring for 16 h, the mixture was allowed to warm up to room temperature. After filtration of the precipitated dicyclohexylurea the filtrate was evaporated and the resulting crude product was recrystallised from isopropanol.

*3.2.2. Synthesis of **8***

A solution of **7** (7.5 mmol) in ethanol (30 ml) was added dropwise to a mixture of L-isoleucine (11.25 mmol) and $NaHCO_3$ (1.9 g) in water (22 ml). The mixture was stirred for 16 h at room temperature and the

organic solvent evaporated. The remaining aqueous solution was acidified (pH 2) by addition of conc. HCl and was extacted with DCM. The combined organic layers were dried over Na_2SO_4 and evaporated under reduced pressure. The residue was recrystallised from EA.

*3.2.3 Synthesis of dipeptide methyl ester (e.g. **9**)*

The N-acylated amino acid (**8**; 10.0 mmol) was dissolved in DCM (60 ml) and DIPCI (10.0 mmol) in DCM (30 ml) was added dropwise at 0 °C over a period of 15 min. After stirring for 30 min a mixture of L-proline methyl ester hydrochloride (12.0 mmol) and DIPEA (14.4 mmol) in DCM (90 ml) was added dropwise (0 °C, 45 min). Stirring over night, evaporation of the solvent, and purification (column chromatography, silica gel, eluent: PE/EA 1 : 1) gave the product.

3.2.4. Hydrolysis of methyl esters

The methyl ester (**9**, **13**, **14**; 5.00 mmol) was stirred in a mixture of aqueous LiOH (1M) and DME (5 ml/5 ml) at rt for 2 h. The mixture was acidified (pH 4) by addition of aqueous citric acid (10%) and extracted with EA. The combined organic layers were dried over Na_2SO_4 and the filtrate evaporated; the residue was chromatographed over silica gel.

3.2.5. Reaction of dipeptides with mono-Boc-protected diaminoalkanes

To an ice-cooled solution of N-acylated dipeptide (6.50 mmol) in DCM (50 ml) a solution of DIPCI (7.15 mmol) in DCM (10 ml) was added dropwise. After stirring for 30 min, a solution of the mono-Boc-protected diaminoalkane (7.15 mmol) in DCM (20 ml) was added dropwise over a period of 15 min. Stirring over night, evaporation of the solvent, and purification (column chromatography, silica gel, eluent: PE/EA/DCM/MeOH 10 : 10 : 10 : 1) yielded the appropriate product.

3.2.6. Conversion of the primary amines into guanidines

Cleavage of the protecting group by means of a TFA/DCM 1 : 1 mixture gave the primary amine (0.64 mmol), which was treated with DIPEA (0.672 mmol) in DCM (10 ml). This solution was stirred for 30 min and – after evaporation of the solvent – the residue was dissolved in a mixture of H_2O and EtOH (5 ml/5 ml) and *S*-methylisothiouronium iodide (0.672 mmol) was added. The mixture was stirred over night, the solvents were evaporated and the residue was purified (column chromatography, silica gel, eluent: EA/DCM/MeOH 2 : 2:1).

3.2.7. Spectroscopic data

All new compounds gave satisfactory ^{1}H NMR, IR, and elemental analysis data in accordance with the assigned structure.
^{1}H NMR data of **RA-1001** (300 MHz, DMSO-d_6, δ [ppm]): 0.73–0.86 (m, 6 H, 2 × CH_3, Ile), 0.90–1.10 (m, 1 H, CH_2, Ile), 1.10–1.30 (m, 1 H, CH_2, Ile), 1.35–1.50 (m, 4 H, CH_2CH_2, agmatine), 1.60–2.10 (m, 5H, β-/γ-CH_2, Pro + β-CH, Ile), 2.36 (t, 2 H, Ar-CH_2CH_2), 2.65 (t, 2 H, Ar-CH_2CH_2), 2.90–3.15 (m, 4 H, $CONHCH_2$ + CH_2-guan.), 3.50 (m, 1 H, δ-CH_2, Pro), 3.72 (m, 1 H, δ-CH_2, Pro), 4.22 (m, 1 H, α-CH, Ile), 4.34 (m, 1 H, α-CH, Pro), 6.62 (d, 2 H, $H_{aromat.}$), 6.96 (d, 2 H, $H_{aromat.}$), 7.60 (m, 1 H, NH, agmatine), 8.03 (m, 1 H, NH, Ile), 8.84 (s, 5 H, guanidyl-HI).
^{1}H NMR data of **RA-1002** (300 MHz, DMSO-d_6, δ [ppm]): 0.93 (d, 6 H, 2 × CH_3, Leu), 1.60–1.95 (m, 9 H, β-CH_2, Leu + γ-CH, Leu + β-CH_2, Pro + γ-CH_2, Pro + CH_2CH_2-guan.), 2.70–2.85 (m, 2 H, $CONHCH_2$), 3.05–3.15 (m, 2 H, CH_2-guan.), 3.45–3.65 (m, 1 H, δ-CH_2, Pro), 3.70–3.90 (m, 1 H, δ-CH_2, Pro), 4.25 (m, 1 H, α-CH, Pro), 4.65 (m, 1 H, α-CH, Leu), 7.37–7.56 (m, 3 H, $H_{aromat.}$), 7.90 (d, 2 H, $H_{aromat.}$), 8.33 (t, 1 H, Pro-NH), 8.48 (s, 5 H, guanidyl-HI), 8.65 (d, 1 H, NH, Leu).

3.3. Inhibition tests

Inhibition tests were carried out according to the following procedure of the thrombin test [12]. The measurements were carried out on a microplate reader (MR 5000, Dynatech, Denkendorf, Germany) at 25 °C. The test medium consisted of 200 µl of Tris buffer (0.05 M; 0.154 M NaCl, 5% ethanol, pH 8.0), 25 µl of aqueous substrate (CH_3SO_2-D-HHT-Gly-Arg-pNA) solution, and 50 µl of enzyme solution. Two concentrations of the inhibitor were used. Three minutes after the addition of the enzyme, 25 µl of acetic acid (50%) was added to quench the reaction, and the optical density was measured at 405 nm. The K_i values reported are means from at least three determinations.

References

1 Radau, G.: Pharmazie **55**, 555 (2000)
2 Trimurtulu, G.; Ohtani, I.; Patterson, G. M. L.; Moore, R. E.; Corbett, T. H.; Valeriote, F. A.; Demchik, L.: J. Am. Chem. Soc. **116**, 4729 (1994)
3 Zhang, L.-H.; Longley, R. E.; Koehn, F. E.: Life Sci. **60**, 751 (1997)
4 Pergament, I.; Carmeli, S.: Tetrahedron Lett. **35**, 8473 (1994)
5 Okino, T.; Matsuda, H.; Murakami, M.; Yamaguchi, K.: Tetrahedron Lett. **34**, 501 (1993)
6 Moore, R. E.; Bornemann, V.; Niemczura, W. P.; Gregson, J. M.; Chen, J.-L.; Norton, T. R.; Patterson, G. M. L.; Helms, G. L.: J. Am. Chem. Soc. **111**, 6128 (1989)
7 Murakami, M.; Ishida, K.; Okino, T.; Okita, Y.; Matsuda, H.; Yamaguchi, K.: Tetrahedron Lett. **36**, 2785 (1995)
8 Sandler, B.; Murakami, M.; Clardy, J.: J. Am. Chem. Soc. **120**, 595 (1998)
9 Matsuda, H.; Okino, T.; Murakami, M.; Yamaguchi, K.: Tetrahedron **52**, 14501 (1996)
10 Murakami, M.; Okita, Y.; Matsuda, H.; Okino, T.; Yamaguchi, K.: Tetrahedron Lett. **35**, 3129 (1994)
11 Steiner, J. L. R.; Murakami, M.; Tulinsky, A.: J. Am. Chem. Soc. **120**, 597 (1998)
12 Stürzebecher, J.; Prasa, D.; Hauptmann, J.; Vieweg, H.; Wikström, P.; J. Med. Chem. **40**, 3091 (1997)

Received April 24, 2002
Accepted June 6, 2002

Dr. Gregor Radau
Department of Pharmaceutical/Medicinal Chemistry
Institute of Pharmacy
The Ernst-Moritz-Arndt-University Greifswald
Friedrich-Ludwig-Jahn-Str. 17
D-17487 Greifswald
radau@pharmazie.uni-greifswald.de