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New Cyanopeptide-Derived Low Molecular Weight Inhibitors of trypsin-like Serine Proteases

This paper deals with the design, syntheses, and inhibition tests of new low molecular weight thrombin inhibitors utilizing cyanopeptides, the secondary metabolites of cyanobacteria with interesting biological activities, as new lead structures. Starting with aeruginosin 98-B (**1**) as a lead structure, we have developed and synthesised new, selective acting inhibitors of serine proteases (**RA-1005** and **RA-1009**), which are suitable targets for further structure-activity studies.

Keywords: Cyanobacteria; Cyanopeptides; Peptide syntheses; Rational drug design; Thrombin inhibitors

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Introduction

Contemporary trends in drug discovery from natural sources favour investigations of the marine environment to yield numerous, often highly complex, chemical compounds. A considerable number of non-toxic cyanopeptides – the secondary metabolites of cyanobacteria (blue-green algae) – inhibit members of the serine proteases family [1] which play central roles in the human organism. Thrombin and factor Xa represent the main actors in the interlaced cycles of blood coagulation and fibrinolysis. A disturbance of the sensitive balance caused by malfunctions of enzymes can result in thromboembolic complications like venous and arterial thrombosis, stroke, restenosis, and recurrent myocardial infarction. The development of oral thrombin inhibitors therefore presents a notable measure for improving the treatment of the above mentioned disorders.

Results and discussion

Structural considerations

Until now, fourteen members of the aeruginosin family have been identified [2–7]. In previous reports, we described the syntheses of the thrombin inhibitors **RA-1001** and **RA-1002** as well as their precursors (**RA-1003** and **RA-1004**) utilizing aeruginosin 98-B – a secondary metabolite from cyanobacterium *Microcystis aeruginosa* – as the starting lead structure (Figure 1) [8–9]. Compounds **RA-1001**, **RA-1002**, and **RA-1003** are equipotent thrombin inhibitors (Table 1). Moreover, **RA-1002**, **RA-1003**, and **RA-1004** seem to be selective inhibitors of thrombin [8].

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X-ray studies of **RA-1001**-trypsin- and **RA-1002**-trypsin-complexes confirm an aeruginosin-analogous binding mode according to the findings described by Sandler et al. [10]. There is a lack of any close interactions of structural elements of aeruginosin 98-B and its synthetic analogues with trypsin's catalytic triad (His57, Asp102, Ser195) – especially concerning residue Ser195. This fact is remarkable, because Ser195 usually attacks the carbonyl group in position P1 of the substrate as a nucleophil ("acylation mechanism", for details see [8] and [10]). Therefore, cyanopeptides of the aeruginosin family as well as their synthetic analogues inhibit trypsin in a non-classical manner. Solely, interactions of positions P1 to P4 with subsites S1 to S4 contribute to the inhibitory power. Agmatines guanidinyl group in the **RA-1001**-trypsin-complex reaches deeply inside the S1-pocket forming a salt bridge to the Asp189-carboxylate side chain. The amide moiety of proline interacts with Gln192 and Ser214. The 3-(4-hydroxyphenyl)propionyl-L-isoleucyl core in **RA-1001** seems to be too flexible to contact with any moiety of trypsin's S3/S4 subsites, what is expressed by a low electron density in the X-ray structure. Compound **RA-1002** shows the same interactions with S1/S2 subsites of trypsin like **RA-1001**. Additionally, the L-leucine unit shows interactions with the backbone amino acid Gly216. The aromatic residues in both inhibitors show no close contact to the enzyme. These findings motivated us to work further on the optimization of the structure/activity relationship of synthetic aeruginosin analogues. In the course of our continuing studies on the development of serine proteases inhibitors we focused our attention on the optimization of the P1-P4 moieties.

Now we report on the synthesis of five new inhibitors (**RA-1005** – **RA-1009**) which were modified in positions P1, P2, P3, and P4 – compared with the lead structures **RA-1001** and **RA-1002**, respectively. **RA-1005** was modified in P2; the L-proline was exchanged for glycine what results in a more flexible conformation. In **RA-1006**

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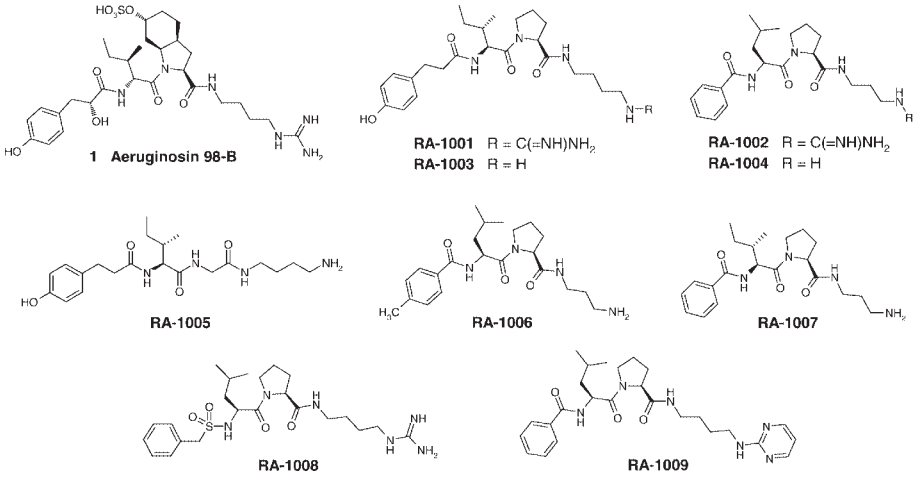


Figure 1. Aeruginosin 98-B (**1**) from *Microcystis aeruginosa* and synthetic analogues **RA-1001** – **RA-1009**.

Table 1. Inhibitory data of synthetic analogues of aeruginosin 98-B.

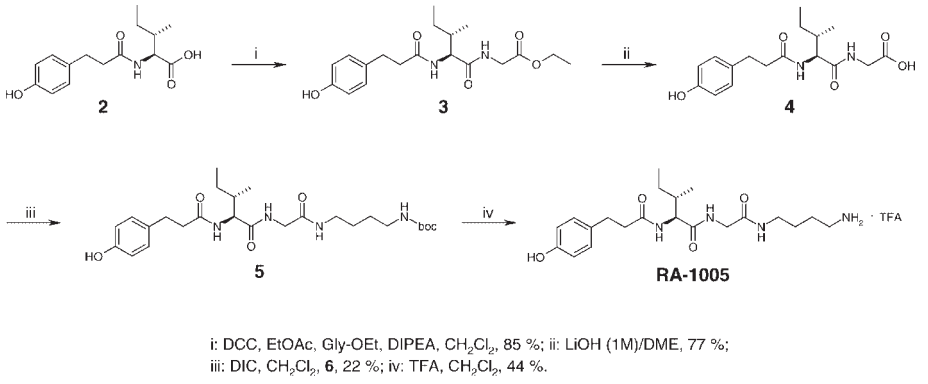
Cp.	K_i (µM)					
	trypsin	thrombin	uPA	plasm	f. Xa	tryptase
RA-1001	32	5.6	>1000	–	>1000	>1000
RA-1002	>1000	8.7	>1000	–	>1000	>1000
RA-1003	>1000	9.0	>1000	–	>1000	>1000
RA-1004	>1000	62	>1000	–	>1000	>1000
RA-1005	54	400	>1000	>1000	>1000	–
RA-1006	>1000	160	–	>1000	>1000	–
RA-1007	>1000	>1000	–	>1000	140	–
RA-1008	5.4	10.6	>1000	–	>1000	–
RA-1009	(19 %)[a]	>1000	>1000	–	>1000	–

uPA = plasminogen activator urokinase, plasm = plasmin, f. Xa = factor Xa, – = not determined.
[a] inhibition at conc. (inhibitor) of 150 µM.

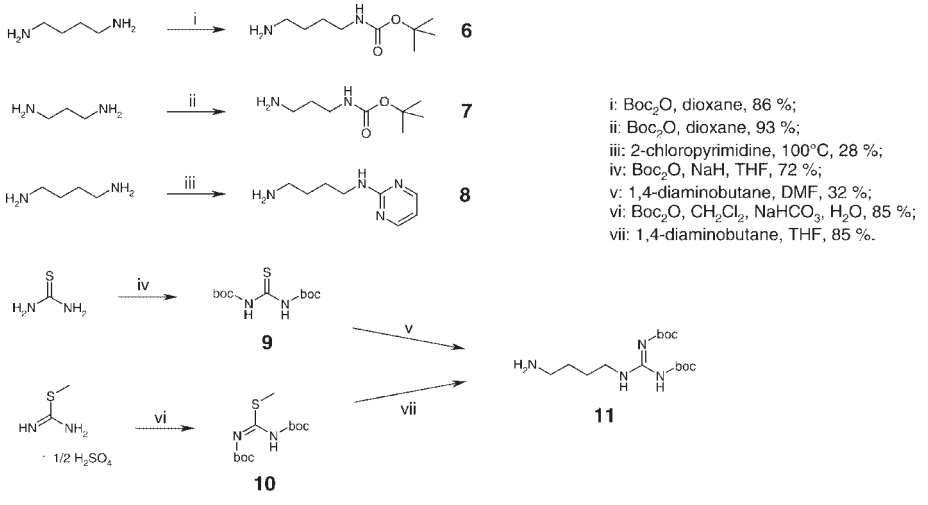
a 4-methylbenzoyl unit was used in P4 instead of the benzoyl group in **RA-1004**, and L-leucine was exchanged for L-isoleucine in P3 of **RA-1007**. In **RA-1008** the phenylmethanesulfonyl moiety was introduced into P4 and the length of the basic side chain in P1 was extended by one carbon atom compared with **RA-1002**. In **RA-1009** we chose a cyclic guanidine equivalent in position P1.

Syntheses

The synthesis of **RA-1005** starts with the synthesis of 3-(4-hydroxyphenyl)propionyl-L-isoleucine (**2**) which was described previously [8] (Scheme 1). Compound **2** reacts in a high-yielded reaction with glycine ethyl ester hydrochloride under DCC-catalysis and basic conditions (DIPEA: diisopropylethylamine; DCC: N,N′-dicyclohexylcarbodiimide). After hydrolysis of the dipeptide ethyl ester (LiOH/DME: 1,2-dimethoxyethane) the resulting acid **4** was connected with the basic side-chain – the mono-Boc-substituted 1,4-diaminobutane (**6**) (Boc: *tert*-butyloxycarbonyl). This step of the synthesis route was the limiting factor in each route of this type. The mono-Boc-substituted diaminoalkanes **6** and **7** were synthesized in analogy to the procedure described by Krapcho [11] in high yields using an eightfold excess of 1,4-diaminobutane and 1,3-diaminopropane referred to di-*tert*-butyl dicarbonate (Boc_2O) in order to avoid the formation of bis-Boc-substituted diaminoalkanes (Scheme 2). Finally, the Boc protecting group was cleaved by means of



Scheme 1. Synthesis of **RA-1005**.

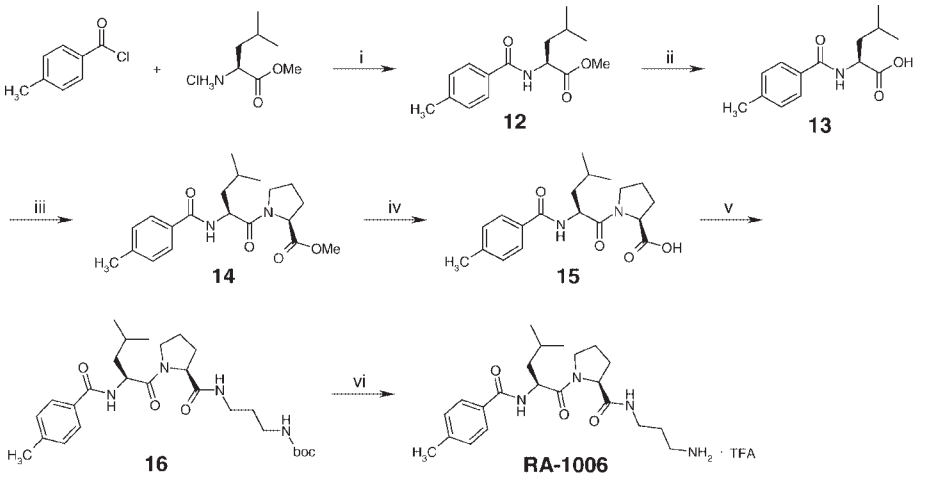


Scheme 2. Syntheses of basic building blocks.

trifluoroacetic acid (TFA) to yield the desired target molecule **RA-1005**.

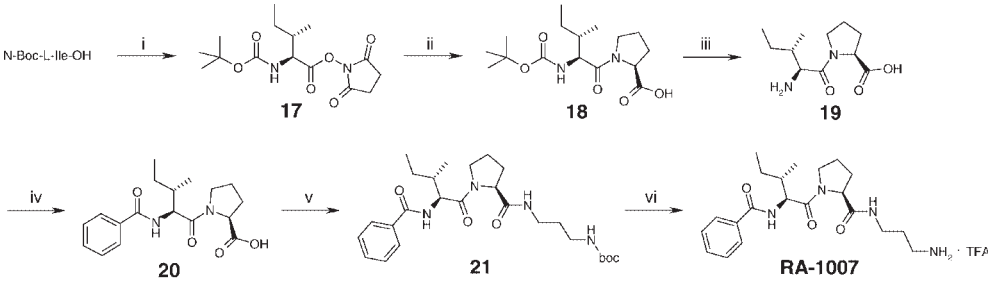
In contrast to the synthesis of **RA-1005** the benzoylation of L-leucine methyl ester hydrochloride was the first step in the preparation of **RA-1006** (Scheme 3). N-(4-Methylbenzoyl)-L-isoleucine methyl ester (**12**) was saponified with LiOH (1M) in dimethoxyethane (DME). Both steps were carried out using alternative procedures which are less complicated compared to the methods described in the literature [12, 13]. The resulting carboxylic acid (**13**) was transformed into the dipeptide methyl ester **14** by condensation with proline methyl ester. Hydrolysis, activation (DIC), and reaction with mono-Boc-substituted diaminopropane (**7**) yielded the appropriate derivative **16**. Boc cleavage was realized in a TFA/dichloromethane mixture and closed this very successful synthesis route to the desired **RA-1006**.

In general terms, the right and most successful synthetic route is very difficult to predict. The synthesis of **RA-1007** for example was more effective following the procedure described in scheme 4 compared with the latter procedure. In the preparation of **RA-1007** the central dipeptide scaffold was synthesised first and the N-terminal benzoyl moiety and the basic C-terminal side chain were introduced in subsequent steps. N-Boc-L-isoleucine was esterificated with N-hydroxysuccinimide (HOSu) under DCC catalysis to its succinimidyl ester **17**, which successfully reacts with completely unprotected L-proline to give the appropriate N-acylated dipeptide **18**. Upon Boc cleavage (TFA/dichloromethane), the completely unprotected L-Ile-L-Pro (**19**) reacted with succinimidyl benzoate with a high yield to the benzoyl derivative **20** under alkaline conditions. The C-terminal basic side chain was introduced into Bz-L-Ile-L-Pro (**20**) according to the procedure described in the synthesis of **RA-1006**.



i: DIPEA, CH_2Cl_2, EtOAc, 77 %; ii: LiOH (1M)/DME, 76 %; iii: DCC, CH_2Cl_2, L-Pro-OMe HCl, DIPEA, 84 %;
iv: LiOH (1M)/DME, 68 %; v: DIC, **7**, CH_2Cl_2, 48 %; vi: TFA, CH_2Cl_2, 46 %.

Scheme 3. Synthesis of **RA-1006**.

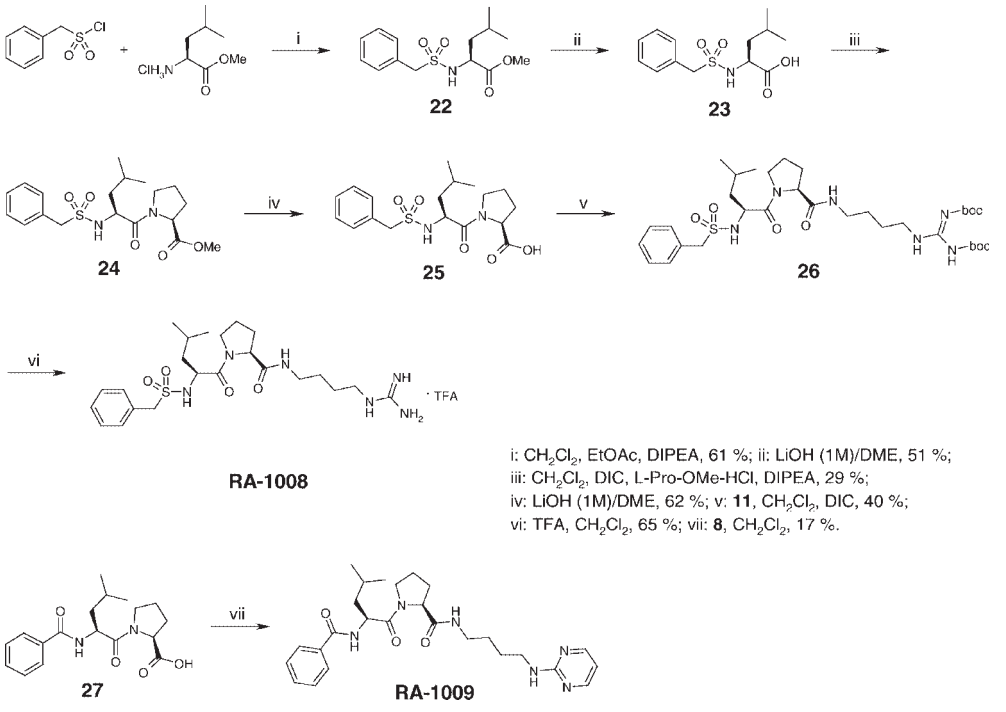


i: DCC, HOSu, EtOAc, 90 %; ii: EtOH, Aceton, L-Pro-OH, $NaHCO_3$, H_2O, 88 %;
iii: TFA, CH_2Cl_2, quant.; iv: PhCOOSu, EtOH, Aceton, $NaHCO_3$, H_2O, 56 %;
v: **7**, DIC, CH_2Cl_2, 38 %; vi: TFA, CH_2Cl_2, 51 %

Scheme 4. Synthesis of **RA-1007**.

Compound **RA-1008** was prepared in an analogous manner as described for the synthesis of **RA-1006** (Scheme 5). Thus, L-leucine methyl ester hydrochloride was acylated by phenylmethanesulfonyl chloride to the appropriate sulfonamide **22**. Treatment of the methyl ester with LiOH (1M)/DME yielded the amino acid **23**. Coupling of **23** with L-proline methyl ester hydrochloride using DIC (N,N′-diisopropylcarbodiimide) and DIPEA in dichloromethane and subsequent saponification of the intermediate product **24** (LiOH/DME) afforded the N-sulfonated dipeptide acid **25**. In contrast to the previously reported syntheses, **25** was condensed with bis-*tert*-butyloxycarbonyl-agmatine (**11**) (DIC/dichloromethane) to the Boc-protected precursor of **RA-1008** (**26**). Treatment of **26** with TFA/dichloromethane gave the guanidine derivative **RA-1008** in satisfactory yields.

Bis-Boc-agmatine (**11**) was prepared via two short syntheses (Scheme 2). Using the first route, the Boc protecting groups were introduced twice in thiourea by means of di-*tert*-butyl dicarbonate – upon deprotonation of thiourea by sodium hydride [14]. Bis-Boc-thiourea (**9**) was subjected to the nucleophilic attack of 1,4-diaminobutane to give the desired bis-Boc-agmatine (**11**) [15]. In a second route, S-methylisothiouronium sulfate was acylated by di-*tert*-butyl dicarbonate in a two-phase-mixture under alkaline conditions to the bis-Boc-substituted derivative **10** [16, 17], which reacted with 1,4-diaminobutane to **11** [18].



Scheme 5. Syntheses of **RA-1008** and **RA-1009**.

Compound **RA-1009** was obtained in very low yield by condensation of the synthetic precursor of **RA-1002**, Bz-L-Leu-L-Pro-OH (**27**) [8], with N-(2-pyrimidinyl)-1,4-diaminobutane (**8**) – a cyclic guaninidine derivative, which was synthesised according to the synthesis of N-(2-pyrimidinyl)-1,3-diaminopropane [19].

Enzyme inhibition tests/Conclusions

The measurements were carried out as described by Stürzebecher et al. [20]; K_i-values were calculated according to Dixon [21] using a linear regression program. Compounds **RA-1001** and **RA-1002** are potent inhibitors of thrombin (K_i 5.6 µM and 8.7 µM, respectively). In contrast to **RA-1001**, aeruginosin 98-B (**1**) inhibits trypsin stronger than thrombin (IC_{50} 0.6 and 10.0 µg/mL [2]). The reason for the differing behaviour may be explained by steric conflicts between aeruginosin's bulky Choi (2-carboxy-6-hydroxy-octahydroindole) group in position P2 and the S2 pocket of thrombin (Tyr60A/Trp60D-loop) which are less prominent in the proline moiety of **RA-1001**. Surprisingly, the synthetic precursor of **RA-1001**, primary amine **RA-1003**, inhibits thrombin in the same order of magnitude (K_i 9.0 µM). None of the four analogues (**RA-1001** – **RA-1004**) decreases the activity of plasminogen activator urokinase (uPA), factor Xa, and human mast cell tryptase.

The P2 moiety of **RA-1005** consists of glycine instead of L-proline. This modification causes a better conformational flexibility, which leads to a loss of selectivity. Compound **RA-1005** inhibits thrombin only weakly, but reacts stronger with trypsin – constrained conformations in position P2 seem to be essential for a selective thrombin inhibition. This is a further example for the fact that effective inhibition of thrombin depends on restricted conformations. Introducing a methylene group into the phenyl ring in P4 of **RA-1004** leads to **RA-1006** which inhibits thrombin certainly slightly weaker than **RA-1004,** but selectively at least. Exchanging L-leucine for L-isoleucine in P3 (**RA-1007**) changes the inhibitory selectivity towards factor Xa. The reasons for this surprising and interesting result have to be clearified by further investigations. Compound **RA-1008** shows a satisfying inhibition of thrombin, but in addition a stronger inhibition of trypsin, too. Comparing the structural features of **RA-1008** with those of **RA-1001**, the loss of selectivity seems to accompany the chain length of four carbon atoms (agmatine) in P1. The phenylmethanesulfonyl moiety in P4 may contribute to the stronger inhibition of trypsin. Compound **RA-1009** shows only a weak inhibition of trypsin (19 % at an inhibitor concentration of 150 µM). The pyrimidine core in P1 does not seem to fit as good as agmatine or noragmatine into the S1 subsite of trypsin-like serine proteases.

In summary, we have developed four equipotent thrombin inhibitors (**RA-1001**, **RA-1002**, **RA-1003**, and **RA-1008**) based on the structure of the cyanopeptide aeruginosin 98-B (**1**). In addition, compounds **RA-1002**, **RA-1003**, **RA-1004**, and **RA-1006** act as selective inhibitors of thrombin, whereas **RA-1001** and **RA-1008** also inhibit trypsin. Compound **RA-1007** was found to be a selective inhibitor of blood coagulation factor Xa, whereas **RA-1006** inhibits thrombin. Further investigations on structure/activity relationships – especially concerning the tranformation of the primary amine derivatives in appropriate guanidine derivatives – are still under way, to complete the results of this study.

Acknowledgements

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Experimental

General

Melting points are not corrected, Mikroheiztisch PHMK 80-2747 (F. Küstner Nachf. KG, Dresden, Germany) – IR spectra (cm^{-1}, KBr or KBr/film in case of oils): IR-Spektrometer Perkin-Elmer 1600 series FTIR (Perkin-Elmer-Deutschland, Rodgau, Germany). – NMR spectra Bruker DPX 200 (200 MHz), NMR spectra Bruker DPX 300 (300 MHz) (Bruker, Rheinstetten, Germany), δ (ppm), solvents: $CDCl_3$, DMSO-D_6, MeOH-D_4, internal standard: TMS (δ = 0.00 ppm). – Elemental analysis: Perkin-Elmer Elemental Analyzer 2400 CHN, all compounds gave satisfactory elemental analyses.- Chromatography: cc: Merck silica gel 60 (0.063–0.200 mm); tlc: Merck aluminium foils silica gel 60 F_{254} (E. Merck, Darmstadt, Germany). – Optical rotation ([α]): Polartronic D (Schmidt Haensch GmbH, Berlin, Germany), determined with Na-D-line (589.3 nm) at 23 °C. – L-Proline methyl ester hydrochloride (**12**) and L-leucine methyl ester hydrochloride (**23**) were prepared by using the thionyl chloride method [22].

Abbreviations of amino acids follow the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature [23]. Other abbreviations: Boc: *tert*-Butyloxycarbonyl, Boc_2O: di-*tert*-butyl dicarbonate, DCC: N,N-dicyclohexylcarbodiimide, DCU: N,N-dicyclohexylurea, DIC: N,N′-diisopropylcarbodiimide, DIPEA: diisopropylethylamine, DME: 1,2-dimethoxyethane, EtOAc: ethyl acetate, HOSu: N-hydroxysuccinimide, PE: petroleum ether, rt: room temperature, TFA: trifluoroacetic acid.

N-[3-(4-Hydroxyphenyl)propionyl]-L-isoleucyl-glycine ethyl ester (**3**)

An ice-cooled solution of 3.00 g N-[3-(4-Hydroxyphenyl)propionyl]-L-isoleucine (10.75 mmol) in dichloromethane (50 mL) was added to a solution of 2.22 g DCC (10.75 mmol) in dichloromethane (20 mL) over a period of 30 min. After stirring for one hour a mixture of 1.65 g glycine ethyl ester hydrochloride (11.83 mmol) and 1.68 g DIPEA (13.01 mmol) in dichloromethane (50 ml) was added dropwise (20 min). While stirring for 16 h, the mixture was allowed to warm up to room temperature. The precipitated DCU was filtered off, the filtrate was evaporated, and the resulting crude product was purified by column chromatography (EtOAc/PE/CH_2Cl_2 1:1:1). Yield: 3.32 g (85 %) of a colourless, amorphous substance. – mp: 98–100 °C – ^{1}H-NMR (200 MHz, $CDCl_3$): δ (ppm) = 0.78–0.90 (m, 6 H, Ile-CH_2-CH_3 + Ile-CH-CH_3), 0.90–1.25 (m, 1 H, Ile-CH_2-CH_3), 1.27 (t, 7.2 Hz, 3 H, OCH_2CH_3), 1.30–1.55 (m, 1 H, Ile-CH_2-CH_3), 1.60–2.00 (m, 1 H, Ile-β-H), 2.45 (dt, 2 H, Ph-CH_2-CH_2), 2.83 (t, 2 H, Ph-CH_2-CH_2), 3.91–4.02 (m, 2 H, Gly-α-H), 4.19 (q, 7.2 Hz, 2 H, OCH_2CH_3), 4.32 (t, 1 H, Ile-α-H), 6.45 (d, 8.8 Hz, 1H, Ile-NH), 6.72 (d, 8.4 Hz, 2 H, $H_{arom.}$), 6.88 (t, 1 H, Gly-NH), 6.97 (d, 8.4 Hz, 2 H, $H_{arom.}$). – IR (KBr, cm^{-1}): 3291, 3083, 2964, 2933, 2875, 1741, 1637, 1546, 1514, 1449, 1371, 1299, 1217, 1101, 1024, 826, 686, 533, 477. – [α] = –31.83 (c = 2 %, MeOH). – $C_{19}H_{28}N_2O_5$ (364.45). C, H, N.

N-[3-(4-Hydroxyphenyl)propionyl]-L-isoleucyl-glycine (**4**)

Ethyl ester **3** (2.86 g, 7.85 mmol) was stirred in a mixture of aqueous LiOH (1 M, 8 mL) and DME (10 mL) at room temperature for 2 h. The mixture was acidified (pH 4) by addition of aqueous citric acid (10 %) and extracted with EtOAc (2 × 30 mL). The combined organic layers were dried with Na_2SO_4 and the filtrate evaporated; the residue was chromatographed over silica gel (eluent: CH_2Cl_2/EtOAc/PE/MeOH 10:10:10:1). Yield: 2.04 g (77 %) of a colourless, amorphous substance. – mp: 61–63 °C – ^{1}H-NMR (300 MHz, DMSO-D_6): δ (ppm) = 0.75–0.82 (d + t, 6 H, Ile-CH_2-CH_3 + Ile-CH-CH_3), 0.95–1.10 (m, 1 H, Ile-CH_2), 1.30–1.40 (m, 1 H, Ile-CH_2), 1.63–1.73 (m, 1 H, Ile-β-H), 2.30–2.50 (m, 2 H, Ph-CH_2-CH_2), 2.64–2.72 (m, 2 H, Ph-CH_2-CH_2), 3.64–3.73 (dd, 17.3 Hz + 5.8 Hz, 1 H, Gly-α-H), 3.74–3.82 (dd, 17.3 Hz + 5.8 Hz, 1 H, Gly-α-H), 4.20 (m, 1 H, Ile-α-H), 6.63 (d, 8.4 Hz, 2 H, $H_{arom.}$), 6.98 (d, 8.4 Hz, 2 H, $H_{arom.}$), 7.82 (d, 9.0 Hz, 1 H, NH), 8.21 (t, 5.9 Hz, 1 H, Gly-NH), 9.09 (s, 1 H, OH), 12.47 (s, 1 H, COOH). – IR (KBr, cm^{-1}): 2500–3400, 3306, 3098, 2965, 2934, 2877, 1731, 1646, 1541, 1515, 1453, 1376, 1220, 1102, 1041, 828, 668, 536. – [α] = –29.93 (c = 1.96 %, MeOH). – $C_{17}H_{24}N_2O_5$ (336.39). C, H, N.

1-(tert-Butyloxycarbonylamino)-4-{N-[3-(4-hydroxyphenyl)propionyl]-L-isoleucyl-glycylamino}butane (**5**)

0.76 g (6.04 mmol) DIC in CH_2Cl_2 (35 mL) was added dropwise to an ice-cooled solution of 1.85 g (5.49 mmol) of **4** in CH_2Cl_2 (60 mL) over a period of 15 min. After stirring for 30 min, a solution of 1.14 g (6.04 mmol) 1-amino-4-[(*tert*-butyloxycarbonyl)amino]butane (**6**) in CH_2Cl_2 (60 mL) was added (15 min). The mixture was stirred for 16 h allowing to warm up to rt and – after evaporation of the solvent – the residue was purified by column chromatography (CH_2Cl_2/PE/EtOAc/MeOH 10:10:10:1). Colourless substance; Yield: 0.60 g (22 %) – mp: 130–131 °C – ^{1}H-NMR (300 MHz, DMSO-D_6): δ (ppm) = 0.79 (m, 6 H, Ile-CH-CH_3 + Ile-CH_2-CH_3), 1.30–1.45 (s + m, 14 H, Boc + NH-CH_2CH_2C$H_2$$CH_2$NHBoc + Ile-C$H_2$-$CH_3$), 1.62–1.72 (m, 1 H, Ile-β-H), 2.30–2.50 (m, 2 H, Ph-CH_2-CH_2), 2.67 (m, 2 H, Ph-CH_2-CH_2), 2.85–2.95 (m, 2 H, CH_2NHBoc), 3.30–3.10 (m, 2 H, Gly-CONH-CH_2), 3.55–3.75 (2× dd, 16.5 Hz + 6.0 Hz bzw. 5.5 Hz, 2 H, Gly-α-H), 4.04 (t, 7.7 Hz, 1 H, Ile-α-H), 6.64 (d, 8.4 Hz, 2 H, $H_{arom.}$), 6.75 (t, 5.4 Hz, 1 H, NH-Boc), 6.99 (d, 8.4 Hz, 2 H, $H_{arom.}$), 7.65 (t, 5.6 Hz, 1 H, Gly-CONH-butyl), 7.96 (d, 7.7 Hz, 1 H, Ile-NH), 8.18 (t, 5.8 Hz, 1 H, Gly-NH), 9.09 (s, 1 H, OH). – IR (KBr, cm^{-1}): 3298, 3082, 2964, 2934, 2873, 1686, 1630, 1542, 1516, 1452, 1366, 1286, 1244, 1170, 828, 694, 534. – [α] = –28.84 (c = 2 %, MeOH) – $C_{26}H_{42}N_4O_6$ (506.65). C, H, N.

1-Amino-4-{N-[3-(4-hydroxyphenyl)propionyl]-L-isoleucyl-glycylamino}butane · TFA (**RA-1005**)

TFA (3 mL) was added to an ice-cooled solution of 0.51 g of **5** (1.00 mmol) in CH_2Cl_2 (15 mL). After stirring at rt for 4 h, the mixture was evaporated in vacuo, dissolved in methanol, and evaporated once again to eliminate residual TFA. The remaining residue was purified by column chromatography (CH_2Cl_2/EtOAc/MeOH 10:10:5). Yield: 0.22 g (44 %) of a colourless, viscous substance. – ^{1}H-NMR (300 MHz, DMSO-D_6): δ (ppm) = 9.16 (s, 1 H, OH), 8.21 (t, 5.8 Hz, 1 H, Gly-NH), 7.98 (d, 7.6 Hz, 1 H, Ile-NH), 7.73–7.80 (s, broad, 4 H, Gly-CON*H*-butyl + NH_3^+), 6.98 (d, 8.4 Hz, 2 H, $H_{arom.}$), 6.64 (d, 8.4 Hz, 2 H, $H_{arom.}$), 4.03 (t, 7.6 Hz, 1 H, Ile-α-H), 3.55–3.75 (2× dd, 16.4 Hz + 6.1 Hz bzw. 5.4 Hz, 2 H, Gly-α-H), 3.00–3.10 (m, 2 H, Gly-CONH-CH_2), 2.75–2.80 (t, 2 H, C$H_2NH_3^+$), 2.68 (t, 7.6 Hz, 2 H, Ph-CH_2-CH_2), 2.35–2.45 (m, 2 H, Ph-CH_2-CH_2), 1.65–1.72 (m, 1 H, Ile-β-H), 1.40–1.55 (m, 5 H, NH-CH_2CH_2C$H_2$$CH_2NH_3^+$ + Ile-CH_2-CH_3), 0.76–0.80 (m, 6 H, Ile-CH-CH_3 + Ile-CH_2-CH_3). – [α] = –22.94 (c = 2 %, MeOH) – $C_{23}H_{35}F_3N_4O_6$ (520.54). C, H, N.

N-(2-Pyrimidinyl)-1,4-diaminobutane (**8**)

13.84 g of DAB (157.30 mmol) were added to 6.00 g of 2-chloropyrimidine (52.40 mmol) and stirred at 100 °C for 6 h. After cooling to rt H_2O (30 mL) was added, the mixture was stirred for 16 h, and additional H_2O (70 mL) was added. To the yellowish solution 4.20 g NaOH (105.00 mmol) in H_2O (20 mL) were added slowly and the solution was stirred for 1 h. The mixture was extracted with dichloromethane (3 × 100 mL), the combined organic layers were dried with Na_2SO_4 and evaporated im vacuo. The resulting residue was recrystallized from PE/EtOAc. Yield: 2.47 g (28 %) of a colourless crystals. – mp: 66–67 °C – ^{1}H-NMR (DMSO-D_6, 300 MHz): δ (ppm) = 1.32 (s, broad, 2 H, NH_2), 1.50–1.60 (m, 2 H, CH_2CH_2-C$H_2$$CH_2NH_2$), 1.60–1.70 (m, 2 H, CH_2C$H_2$$CH_2$$CH_2NH_2$), 2.74 (t, 7.0 Hz, 2 H, CH_2-NH_2), 3.42 (q, 6.8 Hz, 2 H, NH-CH_2), 5.47 (s, broad, 1 H, NH), 8.26 (d, 4.8 Hz, 2 H, H-4/$6_{pyim.}$), 6.50 (t, 4.8 Hz, 1 H, H-$5_{pyim.}$). – IR (KBr, cm^{-1}): 3257, 3108, 2937, 1596, 1512, 1463, 1418, 1370, 1348, 1278, 1230, 1183, 1113, 1088, 1072, 944, 802, 791, 736, 643, 617, 513, 408. – $C_8H_{14}N_4$ (166.23). C, H, N.

N-(4-Methylbenzoyl)-L-leucine methyl ester (**12**)

To a solution of 3.09 g 4-methylbenzoylchloride (20.00 mmol) in EtOAc (100 mL) a mixture of 3.63 g L-leucine methyl ester hydrochloride (20.00 mmol) and 5.81 g DIPEA (45.00 mmol) in EtOAc/dichloromethane (170 mL/40 mL) was added over a period of 40 min at rt. The mixture was stirred for 16 h and the solvent was evaporated to half of the volume and filtered off from DIPEA · HCl. After evaporation to dryness the residue was purified by column chromatography (PE/EtOAc 5:1). Yield: 4.06 g (77 %) of colourless crystals. – mp: 80–81 °C. – ^{1}H-NMR ($CDCl_3$, 300 MHz): δ (ppm) = 0.98 (d, 6.0 Hz, 6 H, CH(CH_3)$_2$), 1.63–1.78 (m, 3 H, C*H*$(CH_3)_2$ + β-CH_2), 2.39 (s, 3 H, CH_3), 3.76 (s, 3 H, OCH_3), 4.82–4.90 (m, 1 H, α-H), 6.54 (d, 8.1 Hz, 1 H, NH), 7.23 (d, 8.3 Hz, 2 H, $H_{arom.}$), 7.70 (d, 8.3 Hz, 2 H, $H_{arom.}$). – IR (KBr, cm^{-1}): 3293, 3066, 3028, 2958, 2360, 1752, 1630, 1545, 1246, 1162. – [α] = –15.50 (c = 2 %, MeOH). – $C_{15}H_{21}NO_3$ (263.34). C, H, N.

N-(4-Methylbenzoyl)-L-leucine (**13**)

Methyl ester **12** (1.44 g, 5.47 mmol) was stirred in a mixture of aqueous LiOH (1 M, 6 mL) and DME (15 mL) at room temperature for 2 h. The mixture was acidified (pH 4) by addition of aqueous citric acid (10 %) and extracted with EtOAc (2 × 20 mL). The combined organic layers were dried with Na_2SO_4 and the filtrate evaporated; the residue was chromatographed over silica gel (eluent: CH_2Cl_2/EtOAc/PE/MeOH 10:10:10:1). Yield: 1.03 g (76 %) of colourless crystals. – mp: 124–126 °C – ^{1}H-NMR (DMSO-D_6, 300 MHz): δ (ppm) = 0.87 (d, 6.3 Hz, 3 H, CH(CH_3)$_2$), 0.92 (d, 6.3 Hz, 3 H, CH(CH_3)$_2$), 1.57–1.77 (m, 3 H, C*H*$(CH_3)_2$ + β-CH_2), 2.35 (s, 3 H, CH_3), 4.39–4.45 (m, 1 H, α-H), 7.27 (d, 8.2 Hz, 2 H, $H_{arom.}$), 7.79 (d, 8.2 Hz, 2 H, $H_{arom.}$), 8.47 (d, 8.0 Hz, 1 H, NH), 12.35 (s, broad, 1 H, COOH). – IR (KBr, cm^{-1}): 2850-3350, 3329, 3043, 2959, 2870, 1728, 1637, 1612, 1532, 1412, 1386, 1231. – [α] = –7.43 (c = 1.93 %, MeOH). – $C_{14}H_{19}NO_3$ (249.31). C, H, N.

N-(4-Methylbenzoyl)-L-leucyl-L-proline methyl ester (**14**)

0.48 g (3.80 mmol) DIC in CH_2Cl_2 (15 mL) was added dropwise to an ice-cooled solution of 0.86 g (3.45 mmol) **13** in CH_2Cl_2 (35 mL) over a period of 30 min. After stirring for further 30 min, a solution of 0.63 g (3.80 mmol) L-proline methyl ester hydrochloride and 0.54 g (4.17 mmol) DIPEA in CH_2Cl_2 (15 mL) was added (40 min). The mixture was stirred for 16 h allowing to warm up to rt and – after evaporation of the solvent – the residue was purified by column chromatography (PE/EtOAc 1:1). Colourless crystals; Yield: 1.05 g (85 %). – mp: 144–147 °C – ^{1}H-NMR ($CDCl_3$, 300 MHz): δ (ppm) = 0.94 (d, 6.4 Hz, 3 H, CH(CH_3)$_2$), 1.05 (d, 6.6 Hz, 3 H, CH(CH_3)$_2$), 1.50–1.65 (m, 3 H, C*H*$(CH_3)_2$ + Leu-β-CH_2), 1.85–2.25 (m, 4 H, Pro-β-CH_2 + Pro-γ-CH_2), 2.39 (s, 3 H, CH_3), 3.56–3.61 (m, 1 H, Pro-δ-CH_2), 3.69 (s, 3 H, OCH_3), 3.74–3.78 (m, 1 H, Pro-δ-CH_2), 4.45–4.55 (m, 1 H, Pro-α-H), 5.05–5.15 (m, 1 H, Leu-α-H), 6.89 (d, 8.7 Hz, 1 H, NH), 7.22 (d, 8.2 Hz, 2 H, $H_{arom.}$), 7.70 (d, 8.2 Hz, 2 H, $H_{arom.}$). – IR (KBr/Film, cm^{-1}): 3323, 2955, 2871, 1749, 1633, 1537, 1436, 1196. – [α] = –42.87 (1.5 %, MeOH). – $C_{20}H_{28}N_2O_4$ (360.45). C, H, N.

N-(4-Methylbenzoyl)-L-leucyl-L-proline (**15**)

Methyl ester **14** (0.94 g, 2.60 mmol) was stirred in a mixture of aqueous LiOH (1 M, 5 mL) and DME (10 mL) at room temperature for 2 h. The mixture was acidified (pH 4) by addition of aqueous citric acid (10 %) and extracted with EtOAc (2 × 20 mL). The combined organic layers were dried with Na_2SO_4 and the filtrate evaporated; the residue was chromatographed over silica gel (eluent: CH_2Cl_2/EtOAc/PE/MeOH 10:10:10:1). Yield: 0.62 g (69 %) of a colourless, viscous substance. – ^{1}H-NMR (DMSO-D_6, 300 MHz): δ (ppm) = 0.90 (d, 6.5 Hz, 3 H, CH(CH_3)$_2$), 1.00 (d, 6.6 Hz, 3 H, CH(CH_3)$_2$), 1.30–1.50 (m, 3 H, C*H*$(CH_3)_2$ + Leu-β-CH_2), 1.65–1.95 (m, 4 H, Pro-β-CH_2 + Pro-γ-CH_2), 2.35 (s, 3 H, CH_3), 3.50–3.65 (m, 1 H, Pro-δ-CH_2), 3.70–3.85 (m, 1 H, Pro-δ-CH_2), 4.45–4.65 (m, 1 H, Pro-α-H), 5.00–5.20 (m, 1 H, Leu-α-H), 7.26 (d, 8.1 Hz, 2 H, $H_{arom.}$), 7.81 (d, 8.1 Hz, 2 H, $H_{arom.}$), 8.60 (2 d, 1 H, NH), 12.45 (s, broad, 1 H, COOH). – IR (KBr, cm^{-1}): 3446, 2800-3500, 3339, 2959, 2872, 1721, 1616, 1386, 1222. – [α] = –37.66 (c = 2 %, MeOH). – $C_{19}H_{26}N_2O_4$ (346.43). C, H, N.

1-(tert-Butyloxycarbonylamino)-3-[N-(4-methylbenzoyl)-L-leucyl-L-prolyl-amino]propane (**16**)

0.20 g (1.58 mmol) DIC in CH_2Cl_2 (10 mL) was added dropwise to an ice-cooled solution of 0.50 g (1.44 mmol) of **15** in CH_2Cl_2 (15 mL) over a period of 10 min. After stirring for 30 min, a solution of 0.27 g (1.58 mmol) 1-amino-3-[(*tert*-butyloxycarbonyl)amino]propane (**7**) in CH_2Cl_2 (20 mL) was added (15 min). The mixture was stirred for 16 h allowing to warm up to rt and – after evaporation of the solvent – the residue was purified by column chromatography (CH_2Cl_2/PE/EtOAc/MeOH 10:10:10:1). Colourless, viscous substance; Yield: 0.36 g (50 %) – ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) = 1.01 (2× d, 6.3 Hz, 6 H, CH(CH_3)$_2$), 1.40 (s, 9 H, Boc), 1.50–1.65 (m, 4 H, Pro-β-CH_2 + Pro-γ-CH_2), 1.70–1.75 (m, 5 H, NH-CH_2C$H_2$$CH_2$NHBoc + C*H*$(CH_3)_2$ + Leu-β-CH_2), 2.40 (s, 3 H, CH_3), 2.95–3.17 (m, 2 H, NH-C$H_2$$CH_2$$CH_2$NHBoc), 3.23–3.29 (m, 2 H, CH_2NHBoc), 3.80–3.90 (m, 1 H, Pro-δ-CH_2), 3.50–

3.60 (m, 1 H, Pro-δ-CH_2), 4.07–4.09 (m, 1 H, Pro-α-H), 4.70–4.77 (m, 1 H, Leu-α-H), 5.27 (t, broad, 1 H, N*H*-Boc), 6.85 (d, 6.9 Hz, 1 H, Leu-N*H*), 7.00 (t, broad, 1 H, Pro-N*H*-CH_2), 7.23 (d, 8.2 Hz, 2 H, $H_{arom.}$), 7.67 (d, 8.2 Hz, 2 H, $H_{arom.}$). – [α] = -32.89 (c = 2 %, MeOH) – $C_{27}H_{43}N_4O_5$ (503.67). C, H, N.

1-Amino-3-[N-(methylbenzoyl)-L-leucyl-L-prolyl-amino]propane · TFA (**RA-1006**)

TFA (3 mL) was added to an ice-cooled solution of 0.32 g of 16 (0.63 mmol) in CH_2Cl_2 (15 mL). After stirring at rt for 4 h, the mixture was evaporated in vacuo, dissolved in methanol and evaporated once again to eliminate residual TFA. The remaining residue was purified by column chromatography (CH_2Cl_2/EtOAc/MeOH 10:10:5). Yield: 0.15 g (46 %) of a colourless, viscous substance. – ^{1}H-NMR ($CDCl_3$, 300 MHz): δ (ppm) = 1.03 (2× d, 6.3 Hz, 6 H, CH(C*H*$_3$)$_2$), 1.50–1.65 (m, 5 H, Pro-β-CH_2 + Pro-γ-CH_2 + C*H*(CH$_3$)$_2$), 1.70–1.85 (m, 4 H, NH-CH_2C*H*$_2$$CH_2$NHBoc + Leu-β-$CH_2$), 2.40 (s, 3 H, CH_3), 2.83–2.87 (m, 2 H, NH-C*H*$_2$$CH_2$-$CH_2NH_2$), 3.20–3.35 (m, 2 H, CH_2CH_2C*H*$_2$$NH_2$), 3.60–3.70 (m, 1 H, Pro-δ-CH_2), 3.95–4.05 (m, 1 H, Pro-δ-CH_2), 4.39–4.42 (m, 1 H, Pro-α-H), 4.70–4.73 (m, 1 H, Leu-α-H), 7.30 (d, 8.3 Hz, 2 H, $H_{arom.}$), 7.78 (d, 8.3 Hz, 2 H, $H_{arom.}$). – [α] = –41.23 (c = 2 %, MeOH) – $C_{24}F_3H_{35}N_4O_5$ (516.55). C, H, N.

N-(tert-Butyloxycarbonyl)-L-isoleucine N-succinimidyl ester (**17**)

To an ice-cooled mixture of 4.80 g N-[*tert*-butyloxycarbonyl]-L-isoleucine (hemihydrate, 20.00 mmol) and 2.30 g HOSu (20.00 mmol) in EtOAc (50 mL) a solution of 4.12 g DCC (20.00 mmol) in EtOAc (20 mL) was added dropwise (30 min). While stirring for 16 h, the mixture was allowed to warm up to room temperature. The precipitated DCU was filtered off, the filtrate was evaporated, and the resulting crude product was recrystallized from *i*-PrOH. Yield: 5.91 g (90 %) of colourless crystals. – mp: 87–88 °C (*i*-PrOH) (lit.: 92–93 °C, *i*-Pr_2O, [24]) – ^{1}H-NMR ($CDCl_3$, 300 MHz,): δ (ppm) = 0.97 (t, 7.4 Hz, 3 H, Ile-CH_2-C*H*$_3$), 1.05 (d, 6.7 Hz, 3 H, Ile-CH-C*H*$_3$), 1.20–1.36 (m, 1 H, Ile-C*H*$_2$-CH_3), 1.46 (s, 9 H, Boc), 1.52–1.67 (m, 1 H, Ile-C*H*$_2$-CH_3), 1.92–2.05 (m, 1 H, Ile-β-H), 2.84 (s, 4 H, OCH_2CH_2O), 4.60–4.65 (m, 1 H, α-H), 5.06 (d, 8.7 Hz, 1 H, NH). – IR (KBr, cm^{-1}): 3371, 3313, 2970, 2936, 2879, 1810, 1784, 1741, 1698, 1529, 1508, 1457, 1425, 1392, 1368, 1308, 1252, 1208, 1166, 1079, 1046, 1014, 910, 873, 813, 648. – $C_{15}H_{24}N_2O_6$ (328.37). C, H, N.

N-(tert-Butyloxycarbonyl)-L-isoleucyl-L-proline (**18**)

A solution of 5.91 g of **17** (18.02 mmol) in EtOH (30 mL) and acetone (10 mL) was added dropwise into a mixture of 3.11 g L-proline (27.03 mmol) and 4.50 g $NaHCO_3$ in H_2O (60 mL) within 30 min. The mixture was stirred for 16 h at rt and the organic solvents were evaporated. The remaining aqueous phase was acidified with conc. HCl (pH 2) and extracted with dichloromethane (3 × 50 mL). The combined organic layers were dried with Na_2SO_4 and evaporated. The crude product was purified by column chromatography (EtOAc/PE/CH_2Cl_2 3:2:1). Yield: 5.22 g (88.0 %) of a colourless, viscous substance. – ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) = 0.89 (t, 6.8 Hz, 3 H, Ile-CH_2-C*H*$_3$), 0.97 (d, 6.7 Hz, 3 H, Ile-CH-C*H*$_3$), 1.08–1.22 (m, 1 H, Ile-C*H*$_2$-CH_3), 1.43 (s, 9 H, Boc), 1.55–1.63 (m, 1 H, Ile-C*H*$_2$-CH_3), 1.70–1.80 (m, 1 H, Ile-β-H), 1.95–2.25 (m, 4 H, Pro-β-CH_2 + Pro-γ-CH_2), 3.60–3.72 (m, 1 H, Pro-δ-CH_2), 3.78–3.88 (m, 1 H, Pro-δ-CH_2), 4.26–4.32 (m, 1 H, Leu-α-H), 4.57–4.61 (m, 1 H, Pro-α-H), 5.30 (d, 9.5 Hz, 1 H, NH), 7.18 (s, broad, 1 H, COOH). – IR (KBr, cm^{-1}): 2600–3500, 3436, 2971, 2934, 2878, 1708, 1636, 1516, 1454, 1392, 1367, 1315, 1251, 1170, 1091, 1044, 1020, 913, 860, 778, 664, 605. – [α] = –85.30 (c = 2.11 %, MeOH) – $C_{16}H_{28}N_2O_5$ (328.41). C, H, N.

L-Isoleucyl-L-proline (**19**)

TFA (8 mL) was added to an ice-cooled solution of 3.09 g (9.42 mmol) of **18** in CH_2Cl_2 (20 mL). After stirring at rt for 3 h, the mixture was evaporated in vacuo, dissolved in methanol and evaporated once again to eliminate residual TFA. The colourless, viscous substance was subjected to reaction with succinimidyl benzoate without further purification. $C_{13}H_{21}F_3N_2O_5$ (342.31).

N-Benzoyl-L-isoleucyl-L-proline (**20**)

A solution of succinimidyl benzoate (1.38 g, 6.28 mmol) in ethanol (30 mL) was added dropwise to a mixture of L-isoleucyl-L-proline (**19**, 3.22 g, 9.42 mmol) and $NaHCO_3$ (1.60 g) in water (20 mL). The mixture was stirred for 16 h at room temperature and the organic solvent was evaporated. The remaining aqueous solution was acidified (pH 2) by addition of conc. HCl and extracted with CH_2Cl_2 (3 × 15 mL). The combined organic layers were dried with Na_2SO_4 and evaporated under reduced pressure. The residue was purified by column chromatography. Yield: 1.82 g (58 %) of a colourless, viscous substance. – ^{1}H-NMR (DMSO-d_6, 300 MHz): δ (ppm) = 0.84 (t, 7.3 Hz, 3 H, Ile-CH_2-C*H*$_3$), 0.97 (d, 6.8 Hz, 3 H, Ile-CH-C*H*$_3$), 1.10–1.20 (m, 1 H, Ile-C*H*$_2$-CH_3), 1.50–1.60 (m, 1 H, Ile-C*H*$_2$-CH_3), 1.80–2.20 (m, 5 H, Pro-β-CH_2 + Pro-γ-CH_2 + Ile-β-H), 3.55–3.65 (m, 1 H, Pro-δ-CH_2), 3.90–4.00 (m, 1 H, Pro-δ-CH_2), 4.21–4.28 (m, 1 H, Pro-α-H), 4.50–4.56 (m, 1 H, Ile-α-H), 7.42–7.52 (m, 3 H, $H_{arom.}$), 7.87–7.88 (m, 2 H, $H_{arom.}$), 8.59 (d, 8.2 Hz, 1 H, NH), 12.30 (s, broad, 1 H, COOH). – IR (KBr, cm^{-1}): 2800-3500, 3316, 2964, 2931, 2877, 1719, 1629, 1534, 1451, 1189, 694. – [α] = –70.18 (c = 2 %, MeOH). – $C_{18}H_{24}N_2O_4$ (332.40). C, H, N.

1-(tert-Butyloxycarbonylamino)-3-(N-benzoyl-L-isoleucyl-L-prolyl-amino)propane (**21**)

0.21 g (1.61 mmol) DIC in CH_2Cl_2 (10 mL) was added dropwise to an ice-cooled solution of 0.49 g (1.46 mmol) of **20** in CH_2Cl_2 (15 mL) over a period of 10 min. After stirring for 30 min, a solution of 0.28 g (1.58 mmol) 1-amino-3-[(*tert*-butyloxycarbonyl)amino]propane (**7**) in CH_2Cl_2 (20 mL) was added (15 min). The mixture was stirred for 16 h allowing to warm up to rt and – after evaporation of the solvent – the residue was purified by column chromatography (CH_2Cl_2/PE/EtOAc/MeOH 10:10:10:1). Colourless, viscous substance; Yield: 0.27 g (38 %) – ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) = 0.91 (t, 7.5 Hz, 3 H, Ile-CH_2-C*H*$_3$), 1.03 (d, 6.6 Hz, 3 H, Ile-CH-C*H*$_3$), 1.43 (s, 9 H, Boc), 1.55–1.70 (m, 4 H, NH-CH_2C*H*$_2$$CH_2$NHBoc + Ile-C*H*$_2$-$CH_3$), 1.85–2.00 (m, 4 H, Pro-β-CH_2 + Pro-γ-CH_2 + Ile-β-H), 2.15–2.25 (m, 1 H, Pro-β-CH_2), 3.00–3.35 (m, 4 H, C*H*$_2$NHBoc + NH-C*H*$_2$$CH_2$$CH_2$NHBoc), 3.65–3.75 (m, 1 H, Pro-δ-CH_2), 3.80–3.95 (m, 1 H, Pro-δ-CH_2), 4.45–4.55 (m, 1 H, Pro-α-H), 4.85–4.90 (m, 1 H, Ile-α-H), 5.31 (t, broad, 1 H, N*H*-Boc), 6.91 (d, 9.0 Hz, 1 H, Ile-N*H*), 7.02 (t, broad, 1 H, Pro-N*H*-CH_2), 7.40–7.51 (m, 3 H, $H_{arom.}$), 7.79–7.82 (m, 2 H, $H_{arom.}$). – [α] = –43.79 (1.18 %, MeOH). – $C_{26}H_{40}N_4O_5$ (488.63). C, H, N.

1-Amino-3-(N-benzoyl-L-isoleucyl-L-prolyl-amino)propane · TFA (**RA-1007**)

TFA (3 mL) was added to an ice-cooled solution of 0.23 g of **21** (0.47 mmol) in CH_2Cl_2 (15 mL). After stirring at rt for 4 h, the mixture was evaporated *in vacuo*, dissolved in methanol, and evaporated once again to eliminate residual TFA. The remaining residue was purified by column chromatography (CH_2Cl_2/EtOAc/MeOH 10:10:5). Yield: 0.12 g (51 %) of a colourless, viscous substance. – ^{1}H-NMR (300 MHz, MeOH-d_4): δ (ppm) = 0.95 (t, 7.4 Hz, 3 H, Ile-CH_2-C*H*$_3$), 1.07 (d, 6.8 Hz, 3 H, Ile-CH-C*H*$_3$), 1.55–1.70 (m, 4 H, NH-CH_2C*H*$_2$$CH_2$$NH_2$ + Ile-C*H*$_2$-CH_3), 1.95–2.05 (m, 4 H, Pro-β-CH_2 + Pro-γ-CH_2 + Ile-β-H), 2.10–2.25 (m, 1 H, Pro-β-CH_2), 2.88 (m, 2 H, NH-C*H*$_2$$CH_2$$CH_2$$NH_2$),

3.15–3.40 (m, 2 H, CH_2NH$_2$), 3.65–3.75 (m, 1 H, Pro-δ-CH$_2$), 4.05–4.15 (m, 1 H, Pro-δ-CH$_2$), 4.35–4.37 (m, 1 H, Pro-α-H), 4.65–4.68 (m, 1 H, Ile-α-H), 7.40–7.55 (m, 3 H, H$_{arom.}$), 7.80–7.85 (m, 2 H, H$_{arom.}$). – IR (KBr, cm^{-1}): 3442, 2927, 1630, 1578, 1540, 1489, 1384, 1316, 714. – [α] = -61.8 (c = 2 %, MeOH) – $C_{23}H_{33}F_3N_4O_5$ (502.53). C, H, N.

N-(Phenylmethanesulfonyl)-L-leucine methyl ester (**22**)

To a solution of 5.72 g phenylmethanesulfonyl chloride (30.00 mmol) in dichloromethane (100 mL) a mixture of 5.46 g L-leucine methyl ester hydrochloride (30.00 mmol) and 8.51 g DIPEA (66.00 mmol) in dichloromethane (150 mL) was added over a period of 45 min at rt. The mixture was stirred for 16 h and after evaporation to dryness the residue was purified by column chromatography (PE/EtOAc 5:1). Yield: 5.45 g (61 %) of a colourless, amorphous substance. – mp: 79–80 °C – ^{1}H-NMR (CDCl$_3$, 300 MHz): δ (ppm) = 0.87 (d, 6.6 Hz, 3 H, CH(CH_3)$_2$), 0.90 (d, 6.6 Hz, 3 H, CH(CH_3)$_2$), 1.40–1.57 (m, 2 H, β-H), 1.61–1.78 (sept., 6.6 Hz, 1 H, CH(CH$_3$)$_2$), 3.73 (s, 3 H, OCH$_3$), 3.88–3.96 (td, 8.8 Hz und 5.8 Hz, 1 H, α-H), 4.23 (d, 13.8 Hz, 1 H, Ph-CH_2SO$_2$), 4.30 (d, 13.8 Hz, 1 H, Ph-CH_2SO$_2$), 4.74 (d, 9.0 Hz, 1 H, NH), 7.34–7.44 (m, 5H, H$_{arom.}$). – IR (KBr, cm^{-1}): 3260, 2958, 2868, 1726 (C=O), 1637, 1495, 1486, 1434, 1409, 1352, 1337, 1325, 1272, 1229, 1201, 1148, 1129, 1096, 989, 948, 910, 824, 783, 702, 609, 547, 524, 474. – [α] = – 30.9 (c = 2 %, MeOH). – $C_{14}H_{21}NO_4S$ (299.39). C, H, N.

N-(Phenylmethanesulfonyl)-L-leucine (**23**)

Methyl ester **22** (3.62 g, 12.10 mmol) was stirred in a mixture of aqueous LiOH (1 M, 12 mL) and DME (20 mL) at room temperature for 2 h. The mixture was acidified (pH 4) by addition of aqueous citric acid (10 %) and extracted with EtOAc (2 × 50 mL). The combined organic layers were dried with Na_2SO_4 and the filtrate evaporated; the residue was chromatographed over silica gel (CH_2Cl_2/EtOAc/PE/MeOH 10:10:10:1). Yield: 1.76 g (51 %) of a colourless, amorphous substance. – mp: 129–132 °C – ^{1}H-NMR (DMSO-d$_6$, 300 MHz): δ (ppm) = 0.85 (d, 6.6 Hz, 6 H, CH(CH_3)$_2$), 1.45 (t, 7.2 Hz, 2 H, β-H), 1.66 (sept., 6.6 Hz, 1 H, CH(CH$_3$)$_2$), 3.75 (dt, 8 Hz, 1 H, α-H), 4.25 (d, 13.8 Hz, 1 H, Ph-CH_2SO$_2$), 4.34 (d, 13.8 Hz, 1 H, Ph-CH_2SO$_2$), 7.33–7.41 (m, 5 H, H$_{arom.}$), 7.57 (d, 8.6 Hz, 1 H, NH), 12.70 (s, broad, 1 H, COOH). – IR (KBr, cm^{-1}): 3174, 2956, 2870, 1744, 1467, 1403, 1313, 1231, 1201, 1147, 1121, 1091, 1015, 966, 937, 915, 847, 790, 735, 698, 683, 607, 568, 524, 464. – [α] = –10.0 (c = 2 %, MeOH) – $C_{13}H_{19}NO_4S$ (285.36). C, H, N.

N-(Phenylmethanesulfonyl)-L-leucyl-L-proline methyl ester (**24**)

1.10 g (8.72 mmol) DIC in CH_2Cl_2 (20 mL) was added dropwise to an ice-cooled solution of 2.26 g (7.93 mmol) **23** in CH_2Cl_2 (90 mL) over a period of 45 min. After stirring for further 30 min, a solution of 1.44 g (8.72 mmol) L-proline methyl ester hydrochloride and 1.24 g (9.59 mmol) DIPEA in CH_2Cl_2 (90 mL) was added (60 min). The mixture was stirred for 16 h allowing to warm up to rt and – after evaporation of the solvent – the residue was purified by column chromatography (PE/EtOAc 5:1). Yield: 0.91 g (29 %) of a colourless, viscous substance. – ^{1}H-NMR (CDCl$_3$, 300 MHz): δ (ppm) = 0.90 (d, 6.6 Hz, 3 H, CH(CH_3)$_2$), 0.91 (d, 6.6 Hz, 3 H, CH(CH_3)$_2$), 1.30–1.45 (m, 2 H, Leu-β-H), 1.80–2.00 (m, 4 H, CH(CH$_3$)$_2$ + Pro-β-CH$_2$ + Pro-γ-CH$_2$), 2.10–2.25 (m, 1 H, Pro-β-CH$_2$), 3.02–3.10 (m, 1 H, Pro-δ-CH$_2$), 3.30–3.37 (m, 1 H, Pro-δ-CH$_2$), 3.72 (s, 3 H, OCH$_3$), 3.80–3.90 (m, 1 H, Pro-α-H), 4.16 (d, 13.9 Hz, 1 H, Ph-CH_2), 4.32 (d, 13.9 Hz, 1 H, Ph-CH_2), 4.43–4.47 (m, 1 H, Leu-α-H), 5.13 (d, 9.1 Hz, 1 H, NH), 7.33–7.46 (m, 5 H, H$_{arom.}$). – IR (KBr/Film, cm^{-1}): 3291, 2964, 2872, 2669, 1743, 1644, 1563, 1455, 1435, 1404, 1364, 1321, 1170, 1131, 1095, 1025, 931, 784, 731, 700, 632, 544. – [α] = – 42.12 (c = 2 %, MeOH) – $C_{19}H_{28}N_2O_5S$ (396.51). C, H, N.

N-(Phenylmethanesulfonyl)-L-leucyl-L-proline (**25**)

Methyl ester **24** (0.90 g, 2.27 mmol) was stirred in a mixture of aqueous LiOH (1 M, 5 mL) and DME (10 mL) at rt for 2 h. The mixture was acidified (pH 4) by addition of aqueous citric acid (10 %) and extracted with EtOAc (2 × 25 mL). The combined organic layers were dried with Na_2SO_4 and the filtrate evaporated; the residue was chromatographed over silica gel (CH_2Cl_2/EtOAc/PE/MeOH 10:10:10:1). Yield: 0.53 g (62 %) of colourless crystals. – mp: 177–179 °C – ^{1}H-NMR (DMSO-d$_6$, 300 MHz): δ (ppm) = 0.85 (d, 6.6 Hz, 6 H, CH(CH_3)$_2$), 1.30–1.40 (m, 2 H, Leu-β-H), 1.65–1.80 (m, 1 H, CH(CH$_3$)$_2$), 1.78–1.95 (m, 3 H, Pro-β-CH$_2$ + Pro-γ-CH$_2$), 2.10–2.20 (m, 1 H, Pro-β-CH$_2$), 3.30–3.60 (m, 2 H, Pro-δ-CH$_2$), 3.85–3.95 (m, 1 H, Leu-α-H), 4.20–4.30 (m, 1 H, Pro-α-H), 4.24 (s, 2 H, Ph-CH_2SO$_2$), 7.30–7.45 (m, 6 H, NH + H$_{arom.}$), 12.33 (s, broad, 1 H, COOH). – IR (KBr, cm^{-1}): 3211, 2958, 2916, 2873, 1719, 1613, 1454, 1430, 1325, 1258, 1154, 1128, 1092, 938, 812, 784, 698, 606, 547, 528, 471. – [α] = –68.83 (c = 2 %, MeOH) – $C_{18}H_{26}N_2O_5S$ (382.48). C, H, N.

1-[N^2,N^3-Bis(tert-butyloxycarbonyl)guanidino]-4-(N-phenylmethanesulfonyl-L-leucyl-L-prolyl-amino)butane (**26**)

178 mg (1.38 mmol) DIC in CH_2Cl_2 (5 mL) was added dropwise to an ice-cooled solution of 479 mg (1.25 mmol) **25** in CH_2Cl_2 (10 mL) over a period of 10 min. After stirring for 30 min, a solution of 497 mg (1.51 mmol) **11** in CH_2Cl_2 (10 mL) was added (10 min). The mixture was stirred for 16 h allowing to warm up to rt and – after evaporation of the solvent – the residue was purified by column chromatography (PE/EtOAc 1:1). Yield: 0.35 g (40 %) of colourless crystals. – mp: 64–66 °C – ^{1}H-NMR (CDCl$_3$, 300 MHz): δ (ppm) = 0.85 (d, 6.6 Hz, 3 H, CH(CH_3)$_2$), 0.87 (d, 6.7 Hz, 3 H, CH(CH_3)$_2$), 1.20–1.30 (m, 2 H, Leu-β-H), 1.40–1.65 (m, 22 H, 2× Boc + NH-CH$_2$CH_2CH_2CH$_2$N$_{guan.}$), 1.80–2.10 (m, 3 H, Pro-β-CH$_2$ + Pro-γ-CH$_2$), 2.15–2.25 (m, 1 H, Pro-β-CH$_2$), 2.95–3.05 (m, 1 H, Pro-δ-CH$_2$), 3.15–3.25 (m, 1 H, Pro-δ-CH$_2$), 3.30–3.40 (m, 2 H, NH-CH_2CH$_2$CH$_2$CH$_2$N$_{guan.}$), 3.75–3.85 (m, 2 H, NH-CH$_2$CH$_2$CH$_2$CH_2N$_{guan.}$), 4.10–4.25 (m, 2 H, Pro-α-H + Ph-CH_2), 4.31 (d, 13.6 Hz, 1 H, Ph-CH_2), 4.35–4.45 (m, 1 H, Leu-α-H), 5.45–5.50 (d, broad, 1 H, Leu-NH), 6.80 (t, broad, 1 H, Pro-NH), 7.30–7.45 (m, 5 H, H$_{arom.}$), 8.28 (s, broad, 1 H, NH$_{guan.}$), 11.45 (s, broad, 1 H, NH$_{guan.}$). – IR (KBr, cm^{-1}): 3340, 2968, 2933, 1720, 1638, 1576, 1456, 1415, 1367, 1331, 1252, 1157, 1133, 1052, 1027, 780, 698, 546. – [α] = –32.66 (c = 2 %, MeOH) – $C_{33}H_{54}N_6O_8S$ (694.88). C, H, N.

1-Guanidino-4-(N-phenylmethanesulfonyl-L-leucyl-L-prolyl-amino)butane · TFA (**RA-1008**)

TFA (1 mL) was added to an ice-cooled solution of 0.24 g of **26** (0.34 mmol) in CH_2Cl_2 (10 mL). After stirring at rt for 5 h, the mixture was evaporated in vacuo, dissolved in methanol and evaporated once again to eliminate residual TFA. The remaining residue was purified by column chromatography (PE/CH_2Cl_2/EtOAc/MeOH 10:10:10:1). Yield: 0.14 g (65 %) of a colourless, viscous substance. – ^{1}H-NMR (DMSO-d$_6$, 300 MHz): δ (ppm) = 0.80 (2× d, 6.5 Hz, 6 H, CH(CH_3)$_2$), 1.30–1.60 (m, 7 H, NH-CH$_2$CH_2CH_2CH$_2$N$_{guan.}$ + Leu-β-H + CH(CH$_3$)$_2$), 1.70–2.05 (m, 4 H, Pro-β-CH$_2$ + Pro-γ-CH$_2$), 3.00–3.10 (m, 1 H, Pro-δ-CH$_2$), 3.10–3.60 (m, 5 H, NH-CH_2CH$_2$CH$_2$CH_2N$_{guan.}$ + Pro-δ-CH$_2$), 3.85–3.90 (m, 1 H, Ile-α-H), 4.05–4.10 (m, 1 H, Pro-α-H), 4.15–4.30 (m, 2 H, Ph-CH_2), 7.25–7.40 (m, 5 H, H$_{aromat.}$), 7.45 (m, 1 H, NH-CH$_2$CH$_2$CH$_2$CH$_2$N$_{guan.}$), 7.90 (s, broad, 1 H, NH, Leu), 8.40 (s, broad, 3 H, guanidine), 8.66 (s, broad, 1 H, guanidine). – IR (KBr/Film, cm^{-1}): 3391, 2964, 1676, 1560, 1458, 1315, 1209, 1142, 844, 801, 723, 699, 604, 546, 520.

– [α] = –43.51 (c = 2 %, MeOH) – $C_{25}F_3H_{39}N_6O_6S$ (608.68). C, H, N.

1-[(Pyrimidin-2-yl)amino]-4-(N-benzoyl-L-leucyl-L-prolyl-amino)butane (**RA-1009**)

116 mg (0.90 mmol) DIC in CH_2Cl_2 (5 mL) was added dropwise to an ice-cooled solution of 285 mg (0.90 mmol) **27** in CH_2Cl_2 (10 mL) over a period of 10 min. After stirring for 30 min, a solution of 164 mg (0.99 mmol) **8** in CH_2Cl_2 (10 mL) was added (10 min). The mixture was stirred for 16 h allowing to warm up to rt and – after evaporation of the solvent – the residue was purified by column chromatography (PE/CH_2Cl_2/EtOAc/MeOH 10:10:10:1). Yield: 72 mg (17 %) of a colourless, viscous substance. – ^{1}H-NMR ($CDCl_3$, 300 MHz): δ (ppm) = 0.90 (d, 6.6 Hz, 3 H, CH(C*H*$_3$)$_2$), 1.04 (d, 6.6 Hz, 3 H, CH(C*H*$_3$)$_2$), 1.55–1.65 (m, 3 H, C*H*(CH$_3$)$_2$ + Leu-β-H), 1.90–2.05 (m, 3 H, Pro-β-CH$_2$ + Pro-γ-CH$_2$), 2.05–2.30 (m, 5 H, Pro-β-CH$_2$ + NH-CH$_2$C*H*$_2$C*H*$_2$CH$_2$N$_{guan.}$), 3.55–3.65 (m, 1 H, Pro-δ-CH$_2$), 3.80–3.90 (m, 1 H, Pro-δ-CH$_2$), 3.85–4.00 (m, 4 H, NH-C*H*$_2$CH$_2$CH$_2$C*H*$_2$NH$_{guan.}$), 4.20–4.30 (m, 1 H, Pro-α-H), 4.50–4.60 (m, 1 H, Leu-α-H), 4.60–4.70 (m, 1 H, NH-CH$_2$CH$_2$CH$_2$CH$_2$N*H*$_{guan.}$), 5.05–5.15 (m, 1 H, N*H*-CH$_2$CH$_2$CH$_2$CH$_2$NH$_{guan.}$), 6.90 (d, 8.2 Hz, 1 H, Leu-NH), 7.45–7.65 (m, 4 H, H$_{arom.}$), 7.70–7.90 (m, 4 H, H$_{arom.}$). – $C_{26}H_{36}N_6O_3$ (480.60). C, H, N.

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