Synthesis of Cyclo-β-tripeptides and Their Biological *in vitro* Evaluation as Antiproliferatives against the Growth of Human Cancer Cell Lines

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Dedicated to the memory of Professor *Luigi M. Venanzi*

A number of cyclo-β-tripeptides and their linear precursors were subjected to primary biological evaluation for cancer-cell growth inhibition (one-dose, three-cell essay), and the five most active ones were then tested in the anti-tumor screen of the *National Cancer Institute* (Bethesda, USA) with 60 human cancer cell lines. Growth inhibition values GI_{50} in the one-digit micromolar, and in one case in the nanomolar range were obtained. The effects show selectivities for certain types of cancer cells and for certain cell lines within these types; the screen includes leukemia, non-small-cell lung, colon, and central-nervous-system (CNS) cancer, melanoma, ovarian, renal, prostate, and breast cancer cell lines. The synthesis and full characterization of two new cyclo-β-peptides, $(\beta^3\text{-HSer(OBn)})_3$ (**11**) and $(\beta^3\text{-HMet})_3$ (**12**) are described. Other cyclo-β-peptides included in this investigation are $(\beta\text{-Asp(Bn)})_3$ (**13**), $(\beta\text{-HGlu(Bn)})_3$ (**14**), and $(\beta\text{-HAla})_3$ (**16**), compounds which had been previously prepared by us. Strongest activities were measured with the cyclo-β-peptides bearing benzyl-ester or benzyl-ether groups in the side chains. The cytotoxic activity of the compounds included in this investigation is much lower ($LC_{50} > 100$ μM) than their antiproliferative activity (GI_{50}).

Introduction. – Oligomers of β-amino acids have recently emerged as a new class of peptidomimetics with a strong folding preference for various secondary structures simply based on different substitution patterns of the β-amino acid building blocks (for reviews, see [1–6]). Over the last five years, all common protein-structural elements such as three different helices [7–14], turns [13–15], pleated sheet, and hairpin structures [10][13][14][16–18] have been identified. More possible structures wait to be elucidated [15][19][20]. β-Peptides are resistant to the degradation by proteinase enzymes [10][21][22] and their building blocks, the β-amino acids are non mutagenic according to *Ames*' tests [21]. Hence, these oligomers may serve as peptidomimetics for the use in medicinal chemistry.

We were the first to demonstrate that β-peptides can possess biological activity by mimicking the peptide hormone *somatostatin* with a *cyclo*-β-tetrapeptide [23][24]. Rational design then led to a *linear* β-tetrapeptide with a 100-fold more potent, nanomolar, selective affinity for the human somatostatin receptor 4 [25]. In addition three other biologically active β-peptides have been designed as cholesterol uptake inhibitors [26] and antibiotics [27–29].

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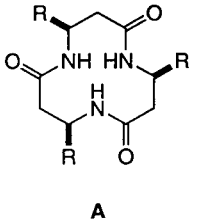
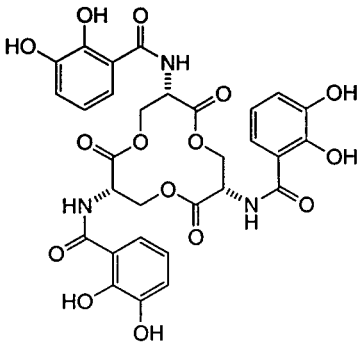
[1]) Part of the dissertation No. 13556 of *K. G.*, ETH-Zürich.

Cyclic β-peptides, however, have received less attention, so far. While cyclo-β-tripeptides[2]) of type **A** (which resemble the bacterial Fe chelator enterobactin (**B**)) have been prepared as long as thirty years ago (from achiral or racemic β-amino acids [33–37]; anthranilic acid [38] and enantiomerically pure β-amino acids [10][39–42]), their structures in the solid state (achiral cyclo(β-HGly) [43]) and in solution [42] have been investigated only recently. Thus, cyclo-β-tripeptides appear to stack to indefinite tube-like hollow structures, so called *nanotubes* in the solid state. These structures are characterized by a *polar hollow center* with apolar side chains. Moreover, due to the unidirectional alignment of the C=O groups, a *large dipole* moment results. In addition, the proper arrangement of the side chains could introduce *molecular function* such as catalysis or binding of metal ions (*cf.* **B**). Hence, we wondered whether those unique properties of cyclo-β-tripeptides could be accompanied by biological activities, *i.e.*, interaction with living cells. We envisioned, for example, inclusion of these compounds in cell membranes or walls, which should result in cytotoxic or antibiotic properties. Hence, a series of cyclo-β-tripeptides was prepared and their antiproliferative properties against cancer cell lines *in vitro* was evaluated.



A



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Synthesis. – There are many methods for the EPC (enantiomerically pure compound) synthesis of β-amino acids. These approaches have been summarized in several review articles [44–47]. The large variety of available α-amino acids [48] renders them ideal starting materials for the synthesis of β^3-amino acids. Hence, the *Arndt-Eistert* homologation of α-amino acids *via* diazo ketones [49] was used as a simple route, on the laboratory scale, with *N*-phtaloyl [50][51] and *N*-carbamate protection [52] on the amino groups. The diazo ketones are decomposed in the *Wolff* rearrangement by using light, heat, or Ag salts to give the ketene intermediates that can be trapped by various nucleophiles [53–57]. When the nucleophile is H_2O, the corresponding β^3-amino acids are isolated, and, with alcohols, the β^3-amino acid esters are formed. An orthogonal protecting-group strategy was selected for β-peptide

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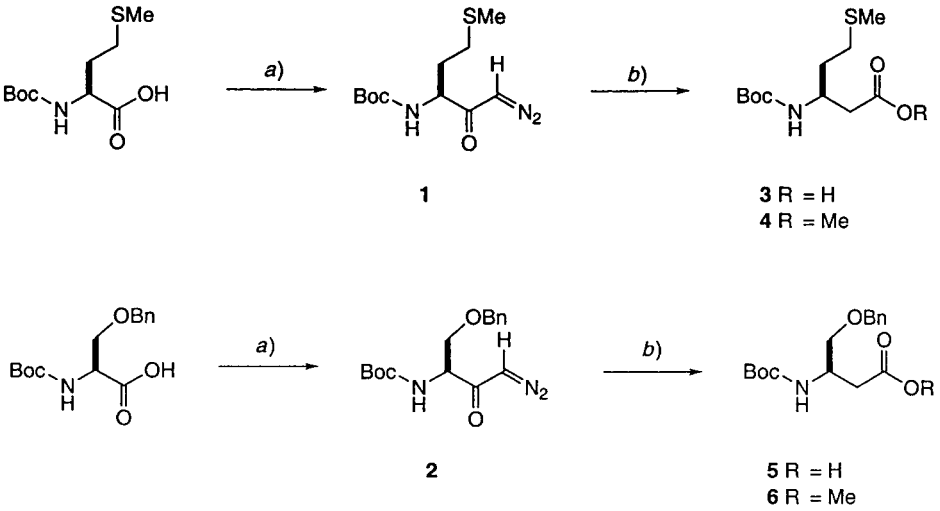
[2]) It is interesting to note that the larger cyclo-β-tetrapeptides have been found to be somatostatin analogues [23][24], molecular scaffolds [30][31] and artificial transmembrane ion channels [32].

synthesis in solution: Boc[3]) for the N-terminus, methylester for the C-terminus, and Bn groups for the side chains.

The homologation of the suitably protected α-amino acids Met and Ser was effected, according to well-established routes [10][53]. In a one-pot procedure, the carboxylic acid functionalities were activated in THF at $-20°$ ($ClCO_2Et$ or $ClCO_2{}^iBu$, Et_3N), and subsequently CH_2N_2[4]) was acylated by the resulting mixed anhydrides to give the diazo esters **1** and **2** (*Scheme 1*). Purification by flash chromatography (for **1**) or by recrystallization and flash chromatography (for **2**) afforded these compounds in yields between 70 and 80%. It is interesting to note that no methylation by the strong alkylating reagent CH_2N_2 of the S-atom of Met was observed under the reaction conditions. This reaction can be conveniently carried out on a multigram scale. Transformation of 0.2 mol of Boc-Met gave 38.8 g of the diazo ketone **1** after flash chromatography.

Scheme 1. *Preparation of β-Amino Acid Derivatives* **3**–**6**



a) $ClCO_2Et$, Et_3N, THF then CH_2N_2, Et_2O. b) For R = H: CF_3CO_2Ag (cat.), Et_3N, THF, H_2O; for R = MeO: CF_3CO_2Ag (cat.), Et_3N, MeOH.

The *Wolff* rearrangement, catalyzed by Ag^+/Et_3N[5]) in a THF/H_2O mixture, gave the β^3-amino acids Boc-β^3-HMet-OH (**3**) and Boc-β^3-HSer(OBn)-OH (**5**) in excellent yields (80–95%). The other two building blocks required, β-amino esters **4** and **6**, were obtained in nearly quantitative yield *via* decomposition of the corresponding diazo esters **1** and **2** in the presence of MeOH as the nucleophile. The cheaper PhCOOAg was used instead of the CF_3COOAg. However, this exchange is only possible, when the products are purified by flash chromatography[6]).

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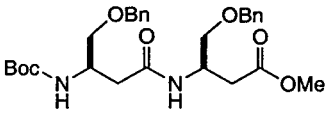
3) For abbreviations, see *Exper. Part*.

4) *Caution:* The generation and handling of CH_2N_2 requires special precautions.

5) The CF_3COO^- counterion is assumed to be exchanged to give the catalytically active Ag^+/Et_3N species.

6) The purification by crystallization is hampered by the high crystallinity of the by-product, benzoic acid.

The synthesis of the β-peptides **7**–**10** was accomplished according to traditional protocols[7]). The Boc group of the β-amino esters **4** and **6** were removed by CF_3COOH in CH_2Cl_2 and subsequent coupling with 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC)/1-hydroxy-1*H*-benzotriazole (HOBt) furnished the β-dipeptides **7** and **9** in high yields. Another cycle of chain elongation resulted in the β-tripeptides **8** and **10**. Both β-peptides have high melting points and low solubility. The higher yield of β-peptide **8** (84%) as compared to **10** (39%) is the result of modified workup procedures[8]). All β-peptides were fully characterized (^{1}H- and ^{13}C-NMR, IR spectroscopy, $[\alpha]_D$, MS spectrometry, and elemental analyses).



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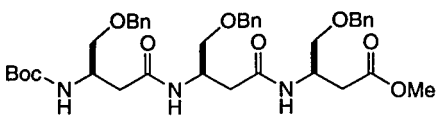

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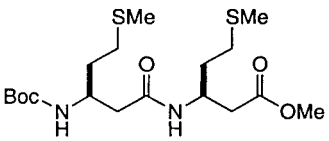

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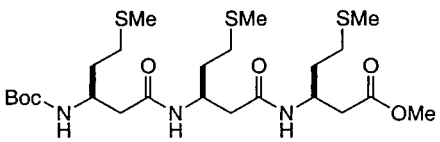

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Having the fully protected β-tripeptide precursors **8** and **10** at hand, it was of importance to choose a clean and efficient cyclization method. There are numerous approaches for the cyclization of α-peptides, which often cyclize in miserable yields. The route developed by *Schmidt et al. via* pentafluorophenyl active esters [58–60] was the method of choice[9]), as it has been previously employed for β-peptides [10]. The very insoluble β-tripeptide **10** could only be dissolved in millimolar concentrations in CF_3CH_2OH, and precipitated partially upon addition of aqueous base. In addition, 50 equiv. of NaOH had to be used in order to cleave the C-terminal methyl ester. After prolonged reaction times, the β-tripeptide acid could be isolated in nearly quantitative yield. Subsequent coupling with pentafluorophenol in DMF using EDC gave the β-peptide active ester, which was terminally *N*-deprotected by CF_3COOH in CH_2Cl_2 to give the trifluoracetate salt. This compound was dissolved in MeCN and added *via* syringe pump to a dilute (3.3 mM) solution of $EtN(i\text{-}Pr)_2$[10]) in MeCN at elevated temperatures (70°) over several hours (*Scheme 2*). The cyclic β-tripeptide **11**

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7) For the solution phase synthesis of other β-peptides carrying functionalized side chaines (Boc/Bn strategy), see, *e.g.*, [41].

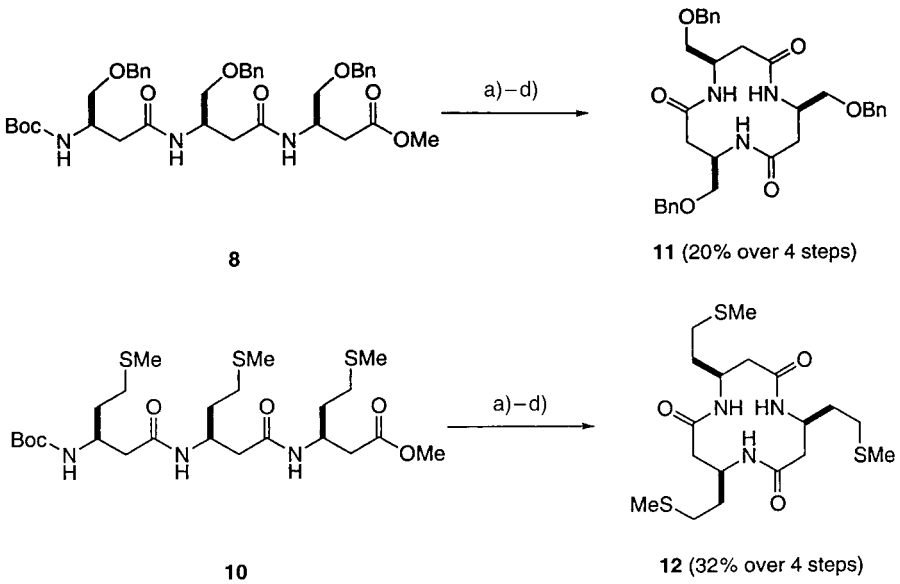
8) Washing the crude β-peptide with a variety of solvents instead of the usual liquid-liquid extraction, and aqueous workup.

9) This method was originally developed for Z-protected peptides. After transformation to the active ester, the Z group was cleaved hydrogenolytically, and the peptide was subsequently cyclized *in situ*. It may be speculated that the metal surface on which the cyclization occurs has a positive effect on the rate and yield of the cyclization [58–60].

10) *Hünig*'s base.

precipitated out of the hot and dilute reaction mixture and could be easily isolated by simple filtration. This β-peptide **11** is insoluble in essentially all common organic solvents, except for TFA. NMR Spectra could only be measured in a mixture of $CDCl_3$ and TFA. In addition, a very high melting point ($>215°$) is observed. The analogous cyclo-β-tripeptide **12** (containing three protected β-HSer residues) was prepared by the same method, albeit in somewhat lower yield, it also has a poor solubility and a high melting point.

Scheme 2. *Deprotection, Activation, and Cyclization to Give the Cyclo-β-tripeptides* **11** *and* **12**



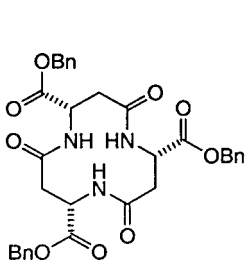
8 **11** (20% over 4 steps)

10 **12** (32% over 4 steps)

a) $NaOH/H_2O/CF_3CH_2OH$. b) C_6F_5OH, EDC, DMF, 0°. c) TFA, CH_2Cl_2. d) Slow addition to a dilute soln. of $EtN(i\text{-}Pr)_2$ in MeCN, 70°.

Biological Evaluation. – To study the influence of the side chains on the antiproliferative activity, as well as the effect of cyclization, several derivatives previously prepared by us were also submitted to the *National Cancer Institute* (*NCI*) for testing. Beside the ether- and thioether containing cyclo-β-tripeptides **11** and **12**, prepared as described above, we submitted the Asp and homologated Glu derived cyclo-β-tripeptides **13** [41] and **14** [41], the protected triolide derived from malic acid **15** [61], as well as $(\beta\text{-HAla})_3$ (**16**) [40], bearing three Me groups as side chains. Finally, the linear precursors **7**–**10** were included in the tests.

All compounds were tested in a first one-dose, three-cell-line assay by the *NCI* (*Table 1*). The *linear* di- and tri-β-peptides **7**, **8**, and **10** displayed antiproliferative activity. Also the cyclo-$(\beta\text{-HSer})_3$ was considered active, whereas the thio derivative cyclo-$(\beta\text{-HMet})_3$ failed to show significant activity under the test conditions. It is interesting to note that the antiproliferative activity is lost when cyclizing the linear, fully protected β-HMet precursor **10** to the cyclo-β-tripeptide **12**. The cyclo-β-peptide



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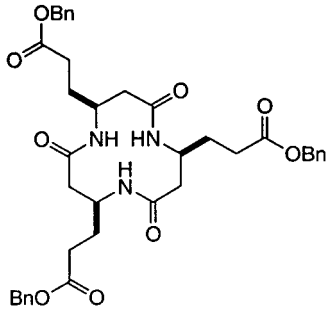

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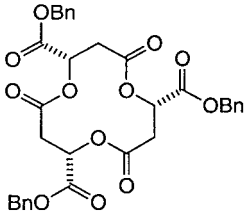

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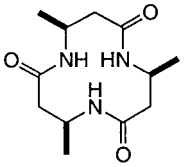

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Table 1. *Primary Biological Evaluation of the Growth Inhibition by Compounds* **7**–**16** *in a One-Dose, Three-Cell Assay.* Compounds showing significant growth inhibition with one cell line at 100 μM concentration were considered active.

Compound	Activity	Compound	Activity
Boc-(β-HSer)$_2$-OMe (**7**)	active	Cyclo(β-HMet)$_3$ (**12**)	inactive
Boc-(β-HSer)$_3$-OMe (**8**)	active	Cyclo(β-Asp)$_3$ (**13**)	active
Boc-(β-HMet)$_2$-OMe (**9**)	inactive	Cyclo(β-HGlu)$_3$ (**14**)	active
Boc-(β-HMet)$_3$-OMe (**10**)	active	Triolide **15**	inactive
Cyclo(β-HSer)$_3$ (**11**)	active	Cyclo(β-HAla)$_3$ (**16**)	inactive

13, derived from the only proteinogenic β-amino acid H_2N-Asp-OH, was shown to inhibit the growth of the cancer cells, and also compound **14** with doubly homologated side chains (an amino adipic acid or β-HGlu derivative). Apparently, amide functionalities in the backbone are required for antiproliferative activity, since replacement of the N- by O-atoms (**13** → **15**) results in complete loss of biological activity. Also the cyclo-β-peptide with methyl side chains was found to be inactive, indicating that a certain functionalization of the side-chains and/or the presence of aromatic Bn groups is necessary for biological activity.

From the results of the preliminary tests, the compounds **7**, **8**, **10**, **11**, **13**, and **14** were chosen for evaluation *in vitro* against 60 human cancer cell lines derived from nine cancer-cell types (leukemia, non-small-cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, renal cancer, prostate cancer, and breast cancer). For each

compound and for each cell line, dose-response curves were obtained with five different concentrations (10-fold dilutions), and the concentration (in μM) causing 50% cell

Table 2. *Growth-Inhibition* (GI_{50}) *Values* (in μM) *of Compounds* **7**, **8**, **10**, **11**, **13**, *and* **14** *Tested in the Anti-Tumor Screen of the* NCI *Consisting of 60 Human Cancer Cell Lines* (NSCLC = non-small-cell lung cancer). All values are the concentrations needed to achieve 50% growth inhibition of the given human cancer cell line. The compounds are tested at a minimum of five concentrations (with 10-fold dilution each). A 48-h continuous drug exposure protocol is used, and a sulforhodamine B protein assay is used to estimate growth relative to a control. The values for β-peptide **13** are the mean values of two independent experiments (except for the CCRF-CEM cell line). The mean values of each cancer cell line were calculated only of the active cell lines ($GI_{50} < 100$ μM), and only if at least three cell lines were thus considered active (n.a. = not available).

	Cell line	**11**	**13**	**14**	**7**	**8**	**10**
Leukemia	CCRF-CEM	>100	0.07	>100	>100	>100	68
	HL-60(TB)	93	0.66	n.d.	>100	>100	1
	K-562	4	4	>100	40	>100	>100
	MOLT-4	6	4	>100	56	>100	>100
	RPMI-8226	>100	39	>100	40	>100	>100
	SR	42	4	>100	39	35	40
	Mean	36	9	>100	44	n.a.	36
NSCLC	EKVX	>100	>100	>100	19	26	35
	HOP-62	18	34	16	21	17	40
	HOP-92	12	21	4	23	25	16
	NCI-H226	>100	>100	>100	>100	>100	42
	NCI-H23	55	47	52	35	>100	47
	NCI-H322M	>100	>100	>100	26	22	30
	NCI-H460	>100	>100	>100	>100	n.d.	69
	NCI-H522	>100	36	>100	21	78	35
	Mean NSCLC	28	35	24	24	33	39
Colon cancer	HCC-2998	>100	91	>100	>100	n.d.	>100
	HCT-116	32	71	26	28	14	37
	HCT-15	>100	>100	>100	31	>100	>100
	HT29	>100	>100	>100	30	72	>100
	KM12	>100	>100	>100	46	>100	>100
	SW-620	>100	>100	>100	56	>100	>100
	Mean Colon	n.a.	n.a.	n.a.	38	43	n.a.
CNS Cancer	SF-268	41	15	n.d.	20	42	20
	SF-295	19	20	9	34	89	22
	SF-539	15	19	6	35	96	17
	SNB-75	3	1	22	25	13	13
	U251	13	12	5	28	93	20
	Mean CNS	18	13	10	28	67	19
Melanoma	LOX IMVI	>100	>100	>100	53	59	>100
	M14	>100	87	>100	33	55	58
	SK-MEL2	28	13	17	37	>100	24
	SK-MEL28	>100	>100	>100	>100	72	>100
	SK-MEL-5	>100	>100	>100	46	>100	56
	UACC-257	>100	>100	>100	>100	>100	69
	Mean	n.a.	n.a.	n.a.	42	62	52

Table 2 *(cont.)*

	Cell line	**11**	**13**	**14**	**7**	**8**	**10**
Ovarian	IGROVI	> 100	98	50	34	100	56
	OVCAR-3	81	> 100	98	28	100	22
	OVCAR-4	> 100	> 100	> 100	19	4	20
	OVCAR-5	> 100	> 100	> 100	34	100	> 100
	OVCAR-8	> 100	> 100	> 100	25	24	29
	SK-OV-3	20	21	6	23	60	20
	Mean ovarian	50	n.a.	51	27	65	30
Renal cancer	768-0	17	21	8	30	18	34
	A498	12	28	8	27	24	21
	ACHN	17	23	17	39	81	37
	CAKI-1	> 100	> 100	> 100	30	> 100	> 100
	RXF 393	11	13	2	30	21	16
	TK-10	18	20	6	> 100	n.d.	19
	UO-31	> 100	> 100	> 100	32	> 100	> 100
	Mean renal	15	21	8	31	36	25
Prostate	PC-3	> 100	> 100	> 100	27	13	> 100
	DU-145	> 100	> 100	> 100	22	23	87
	Mean prostate	> 100	> 100	> 100	25	18	n.a.
Breast cancer	MCF 7	> 100	> 100	> 100	20	12	38
	NCI/ADR-RES	> 100	> 100	> 100	22	> 100	> 100
	MDA-MB-231	27	14	14	37	81	39
	HS 578T	40	13	5	26	35	30
	MDA-MB-435	> 100	> 100	> 100	34	33	40
	MDA-N	> 100	> 100	> 100	52	> 100	69
	T-47D	62	> 100	45	34	25	63
	Mean breast	43	n.a.	21	32	37	47

growth inhibition (GI_{50}) relative to the control was calculated. The results are collected in *Table 2*.

The cyclo(β-HSer)$_3$ (**11**) displays moderate antiproliferative activity against the leukemia cell lines K-562 (4 μM) and MOLT-4 (6 μM), against several CNS cancer cell lines (3 μM for SNB-75, mean 18 μM), and against renal cancer (mean 15 μM), whereas only weak or no activity was measured for most of the other cell lines.

However, its derivative cyclo(Asp(OBn))$_3$ (**13**) possesses *strong growth inhibiting properties* against several leukemia cell lines. *Submicromolar values* have been measured for CCRF-CEM (0.07 μM) and for HL-60 TB (0.66 μM). In addition, this compound shows moderate-to-good antiproliferative activities *in vitro* against CNS cancer (SNB-75 cell line with 1 μM, mean 13 μM) and renal cancer cell lines. Again, only weak to no activity has been determined with **13** for *in vitro* activity against colon cancer, melanoma, ovarian, and several breast cancer cell lines, indicating a certain selectivity in this assay.

The doubly homologated Glu derived cyclo-β-peptide **14**, sharing the BnOCO groups with **13**, displays no antiproliferative activity against leukemia cell lines. However, the cyclotripeptide **14** exhibits activity against CNS (mean 10 μM) and renal (mean 8 μM) cancer cell lines, which is stronger than that observed with **13**.

Furthermore, some good values were recorded for some non-small-cell lungs cancer (HOP-92, 4 μM), ovarian (SK-OV-3, 6 μM), and breast (HS 578T, 5 μM) cancer cell lines, whereas only weak or no activity was found in the prostate, melanoma, and colon cancer tests.

To our surprise, the linear precursors tested did show a broad, yet weak-to-moderate activity against many of the cell lines tested. Thus, the dipeptide **7** has the broadest, *i.e.*, least selective activity (it was considered active for 52 of 60 cell lines evaluated), with no high values having been found. The longer-chain linear tripeptides **8** and **10** appear to be more selective (compare the values obtained for **7** and **8** for leukemia, and of **7** and **10** for colon cancer cell lines). In addition, some good activities have been detected for the linear peptides **8** (OVCAR-4, 4 μM) and for **10** (HL-60 TB, 1 μM).

The β-peptides included in this investigation have also been tested for cytotoxic activity; *ca.* 90% of the LC_{50} values were > 100 μM, *i.e.*, outside of the measuring range of the test. This means that the recorded antiproliferative activities are selective against human cancer cells!

Conclusion. – Several β-peptides have been evaluated *in vitro* as antiproliferatives against the growth of human cancer cell lines. It is interesting to see that several cyclic tri-β-peptides, as well as two β-dipeptides and a β-tripeptide, display antiproliferative activity by inhibiting the growth of several cancer cell lines. The β-peptide cyclo(β-Asp)$_3$ **13** is the most potent compound in this series displaying *submicromolar* activity (GI_{50} values down to 70 nM) against two leukemia cell lines. This compound appears to inhibit preferentially the growth of leukemia, CNS, and renal cell lines, and only little or no activity has been found for the other cell lines evaluated.

The presence of an amide backbone in the cyclo-β-tripeptides as well as of Bn groups appear to be necessary for biological activity. In addition, it looks like cyclization is enhancing the growth inhibition effect.

The antiproliferative properties of some of the evaluated β-peptides are an additional demonstration of a biological activity of β-peptides. Given the remarkable, complete stability of β-peptides against microbial, fungal, and mammalian peptidases [22], the spectrum of their biological activity is now broadened from mimicking a natural α-peptidic hormone [23–25], inhibiting a protein receptor [26], and having antibacterial activity [27–29], to inhibiting the growth of human cancer cells.

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Experimental Part

1. *General*. Abbreviations: Boc: (*tert*-butoxy)carbonyl, DCC: 1,3-dicyclohexylcarbodiimide, EDC: 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride, FAB: fast atom bombardment, FC: flash chromatography, HOBt: 1-hydroxy-1*H*-benzotriazole, h.v.: high vacuum, 0.01–0.1 Torr, β-HXxx: β-homoamino acid, NMM: *N*-methylmorpholine, TFA: CF_3COOH, TFE: 2,2,2-trifluoroethanol. THF was freshly distilled

over K under Ar before use. Et_3N was distilled over CaH_2 and stored over 4-Å molecular sieves. Solvents for chromatography and workup procedures were distilled from *Sikkon* (anh. $CaSO_4$; *Fluka*). Boc-Ser(OBn)-OH was purchased from *Senn Chemicals*. Boc-Met-OH was prepared from Met [62]. Compounds **13**, **14** [41], **15** [61], and **16** [40] were prepared according to the literature procedures. All other reagents were used as received from *Fluka*. TLC: *Merck* silica gel *60* F_{254} plates; detection with UV or dipping into a soln. of anisaldehyde (9.2 ml), AcOH (3.75 ml), conc. H_2SO_4 (12.5 ml), and EtOH (338 ml), followed by heating. FC: *Fluka* silica gel *60* (40–63 mm); at *ca.* 0.3 bar. Anal. HPLC: *Knauer* HPLC system (pump type *64*, *EuroChrom 2000* integration package, degaser, UV detector (variable-wavelength monitor)), *Macherey-Nagel* C_8 column (*Nucleosil 100-5* C_8 (250 × 4 mm)). Prep. HPLC: *Knauer* HPLC system (pump type *64*, programmer *50*, UV detector (variable-wavelength monitor)), *Macherey-Nagel* C_8 column (*Nucleosil 100-7* C_8 (250 × 21 mm)). M.p.: *Büchi 510* apparatus; uncorrected. Optical rotations: *Perkin-Elmer 241* polarimeter (10 cm, 1 ml cell) at r.t. IR Spectra: *Perkin-Elmer 782* spectrophotometer. NMR Spectra: *Bruker AMX-500* (^{1}H: 500 MHz, ^{13}C: 125 MHz), *AMX-400* (^{1}H: 400 MHz, ^{13}C: 100 MHz), *ARX-300* (^{1}H: 300 MHz), *Varian Gemini-300* (^{1}H: 300 MHz, ^{13}C: 75 MHz), or *Gemini-200* (^{1}H: 200 MHz, ^{13}C 50 MHz); chemical shifts (δ) in ppm downfield from Me_4Si (= 0 ppm); J values in Hz. MS: *VG Tribrid* (EI) or *Hitachi Perkin-Elmer RHU-6M* (FAB in a 3-nitrobenzyl-alcohol matrix) spectrometer; in m/z (% of basis peak). Elemental analyses were performed by the Microanalytical Laboratory of the Laboratorium für Organische Chemie der ETH-Zürich.

2. *Preparation of Diazo Ketones. General Procedure 1* (*GP 1*). *Caution:* The generation and handling of CH_2N_2 requires special precautions [63][64]. The *N*-Boc-protected α-amino acid was dissolved in THF (0.2M) under Ar. The soln. was cooled to $-15°$, and Et_3N (1 equiv.) and ClC(O)OEt or ClC(O)O^{i}Bu (1 equiv.) were added. After 15 min, the suspension was allowed to warm to 0°, and a soln. of CH_2N_2 in Et_2O was added until a strong yellow color persisted. The suspension was allowed to warm to r.t. and stirred for 3 h. The excess of CH_2N_2 was destroyed by the addition of few drops of AcOH. The mixture was diluted with H_2O, and the bulk of THF was evaporated. After addition of Et_2O, the mixture was washed with sat. $NaHCO_3$, NH_4Cl, and NaCl solns. The org. layer was dried ($MgSO_4$) and evaporated. FC or recrystallization gave the pure diazo ketones.

3. *Homologation of the Diazo Ketones to the Corresponding β-Amino Acids. General Procedure 2* (*GP 2*). The diazo ketone was dissolved in THF/H_2O 9:1 (*v*/*v*) (0.25M). Under the exclusion of light, the soln. was cooled to *ca.* $-25°$, and a soln. of CF_3CO_2Ag (0.11 equiv.) in Et_3N (2.3 equiv.) was added. The mixture was allowed to warm to r.t. and stirred for 3 h in the dark. THF was evaporated, and the residue was dissolved in Et_2O. The β-amino acid was extracted with sat. $NaHCO_3$ soln., and the H_2O layer was carefully acidified at 0° to pH 2–3 and extracted with Et_2O. The combined org. layers were dried ($MgSO_4$) and evaporated. The resulting β-amino acids were used without further purification.

4. *Homologation of the Diazo Ketones to the Corresponding β-Amino Acid Methyl Esters. General Procedure 3* (*GP 3*). The diazo ketone was dissolved in MeOH (0.25M). Under the exclusion of light, the soln. was cooled to *ca.* $-25°$, and a soln. of CF_3CO_2Ag or $PhCO_2Ag$ (0.11 equiv.) in Et_3N (2.3 equiv.) was added. The mixture was allowed to warm to r.t. and stirred for 3 h in the dark. The solvent was evaporated and the residue was dissolved in Et_2O. The org. layer was washed with sat. $NaHCO_3$, NH_4Cl and NaCl solns., dried ($MgSO_4$), and evaporated.

5. *Boc-Deprotection with TFA. General Procedure 4* (*GP 4*). The Boc-protected compound was dissolved in CH_2Cl_2 (0.25M) and cooled to 0°. The same amount (*v*/*v*) of TFA was added, and the mixture was stirred for 1 h at 0° and for 1 h at r.t. The solvent was evaporated, the residue was twice taken up in $CHCl_3$ and evaporated. The resulting trifluoroacetate was dried under h.v. and used without further purification.

6. *Peptide Coupling with EDC and HOBt. General Procedure 5* (*GP 5*). The trifluoroacetate of the amino fragment was dissolved in $CHCl_3$ (0.5M) and cooled to 0°. Et_3N (4 equiv.), HOBt (1.2 equiv.), a soln. of the Boc-protected fragment (1 equiv.) in $CHCl_3$ (0.25M), and EDC (1.2 equiv.) were added, and the resulting mixture was stirred for 30 min at 0°. The mixture was then allowed to warm to r.t. and stirred for additional 12 h (TLC control). The mixture was diluted with $CHCl_3$ and was washed with 1M HCl, sat. $NaHCO_3$, and sat. NaCl solns. The org. layer was dried ($MgSO_4$) and evaporated, and the resulting peptides were either purified or used without further purification.

tert-*Butyl* N-*{(1S)-3-Diazo-1-[2-(methylsulfanyl)ethyl]-2-oxopropyl}carbamate* (**1**). Boc-Met-OH (49.9 g, 0.2 mol) was transformed according to *GP 1*. FC (Et_2O/pentane 1:1) gave compound **1** (38.8 g, 71%). Yellow solid. M.p. 62.5–63.5°. R_f (Et_2O/pentane 1:1) 0.20. $[\alpha]_D = -16.7$ ($c = 1.03$). IR ($CHCl_3$): 858*w*, 1020*w*, 1045*w*, 1163*s*, 1369*s*, 1443*w*, 1496*s*, 1641*s*, 1708*s*, 2111*s*, 2920*w*, 2981*m*, 3008*m*, 3432*w*. ^{1}H-NMR (300 MHz, $CDCl_3$): 1.43 (*s*, *t*-Bu); 1.79–2.13 (*m*, $MeSCH_2CH_2$); 2.10 (*s*, Me); 2.54 (*t*, $J = 7.3$, $MeSCH_2CH_2$); 4.32 (br., CH); 5.23 (br. *d*, $J = 7.5$, NH); 5.50 (br. *s*, CHN_2). ^{13}C-NMR (75 MHz, $CDCl_3$): 15.47; 28.32; 30.10; 32.00; 54.20; 56.47; 80.17;

155.36; 193.35. EI-MS: 199 (35, $[M - t\text{-BuOH}]^+$), 189 (22), 172 (26, $[M - \text{Boc}]^+$), 161 (31), 157 (22), 146 (53), 143 (50), 115 (20), 104 (43), 61 (41), 57 (100). Anal. calc. for $C_{11}H_{19}N_3O_3S$ (273.11): C 48.33, H 7.01, N 15.37; found: C 48.42, H 7.02, N 15.26.

tert-*Butyl* N-*{(1S)-[(Benzyloxy)methyl]-3-diazo-2-oxopropyl}carbamate* (**2**). Boc-Ser(OBn)-OH (33.5 g; 113 mmol) was transformed according to *GP 1*. Recrystallization (AcOEt/$(i\text{-Pr})_2$O/pentane) and FC (Et_2O/pentane 1:2 to 1:1) gave compound **2** (28.6 g, 79%). Yellow needles. M.p. 81–83° ([41]: 87–88°). $[\alpha]_D = -23.6$ ($c = 1.00$, $CHCl_3$). ([41]: $= -24.2$ ($c = 1.13$, $CHCl_3$)). NMR Data in agreement with those in [41].

*(*S*)-3-{[(*tert*-Butoxy)carbonyl]amino}-5-(methylsulfanyl)pentanoic Acid* (**3**). Diazo ester **1** (2.0 g, 7.31 mmol) was transformed according to *GP 2*. Recrystallization (AcOEt) gave compound **3** (1.6 g, 83%). Off-white crystals. M.p. 82.5–83.5°. $[\alpha]_D = -19.4$ ($c = 1.03$). IR ($CHCl_3$): 850*w*, 928*w*, 1050*w*, 1166*s*, 1368*m*, 1393*w*, 1503*s*, 1708*s*, 2930*w*, 2981*m*, 3008*m*, 3435*w*. ^{1}H-NMR (300 MHz, $CDCl_3$): 1.44 (*s*, *t*-Bu); 1.76–1.95 (*m*, $MeSCH_2CH_2$); 2.10 (*s*, Me); 2.17–2.61 (*m*, $MeSCH_2CH_2$, CH_2CO); 4.01 (br., CH); 5.05 (br. *d*, $J = 8.1$, NH); 5.97 (br., OH). ^{13}C-NMR (75 MHz, $CDCl_3$): 15.56; 28.35; 30.75; 33.88; 38.88; 46.74; 79.75; 155.49; 176.55. MS: 547 (30, $[2M + \text{Na}]^+$), 525 (44, $[2M + \text{H}]^+$), 262 (100, $[M - \text{H}]^+$). Anal. calc. for $C_{11}H_{21}NO_4S$ (263.35): C 50.17, H 8.04, N 5.32; found: C 50.24, H 7.83, N 5.32.

*Methyl (*S*)-3-{[(*tert*-Butoxy)carbonyl]amino}-5-(methylsulfanyl)pentanoate* (**4**). Diazo ester **1** (10.0 g; 36.59 mmol) was transformed according to *GP 3*. FC (Et_2O/pentane 2:3), subsequent thorough drying under h.v., and storing at −20° gave compound **4** (9.6 g, 95%). Colorless needles. R_f (Et_2O/pentane 2:3) 0.28. M.p. 36–37°. $[\alpha]_D = -22.1$ ($c = 1.12$). IR ($CHCl_3$): 1054*w*, 1166*s*, 1289*w*, 1368*m*, 1392*w*, 1438*m*, 1501*s*, 1707*s*, 2981*m*, 3008*m*, 3435*w*. ^{1}H-NMR (300 MHz, $CDCl_3$): 1.41 (*s*, *t*-Bu); 1.75–1.84 (*m*, $MeSCH_2CH_2$); 2.08 (*s*, MeS); 2.43–2.58 (*m*, $MeSCH_2CH_2$, CH_2CO); 3.66 (*s*, MeO); 3.96–4.04 (*m*, CH); 5.02 (*d*, $J = 8.4$, NH). ^{13}C-NMR (75 MHz, $CDCl_3$): 15.54; 28.35; 30.76; 34.01; 38.90; 46.91; 51.71; 79.41; 155.29; 171.92. EI-MS: 221 (24), 157 (100), 129 (47), 128 (51), 102 (28), 61 (28), 59 (26). Anal. calc. for $C_{12}H_{23}NO_4S$ (277.38): C 51.96, H 8.36, N 5.05; found: C 51.83, H 8.51, N 5.06.

*(*R*)-4-(Benzyloxy)-3-{[(*tert*-butoxy)carbonyl]amino}butanoic Acid* (**5**). Diazo ester **2** (12.72 g; 40 mmol) was transformed according to *GP 2*. Drying under h.v. gave compound **5** (9.6 g; 78 %). White crystals. M.p. 104–106°. ([41]: 73–74°). ^{1}H-NMR (200 MHz, $CDCl_3$): in agreement with those in [41].

*Methyl (*R*)-4-(Benzyloxy)-3-[(*tert*-butoxy)carbonyl]amino}butanoate* (**6**). Diazo ester **2** (24.4 g, 76.4 mmol), 1.92 g (8.4 mmol) of $PhCO_2Ag$, and 30.8 ml (220 mmol) of Et_3N were transformed according to *GP 3*. FC (Et_2O/pentane 1:2) gave **6** (22.63 g; 92%). Colorless solid. R_f (Et_2O/pentane 2:3) 0.47. M.p. 34–35°. $[\alpha]_D = -12.7$ ($c = 2.90$, $CHCl_3$). ^{1}H-NMR (300 MHz, $CDCl_3$): 1.43 (*s*, *t*-Bu); 2.62 (*d*, $J = 6.5$, CH_2CO_2Me); 3.48–3.63 (*m*, CH_2O, MeO); 4.16 (br., CHN); 4.50 (*s*, $PhCH_2$); 5.18 (br., NHBoc); 7.24–7.36 (*m*, 5 arom. H). ^{13}C-NMR (300 MHz, $CDCl_3$): 28.37; 36.15; 47.28; 51.63; 71.03; 73.19; 79.45; 127.63; 127.73; 128.39; 137.92; 155.92; 171.94. IR ($CHCl_3$): 3675*w*, 3613*w*, 3487*w*, 3446*w*, 3026*w*, 2974*w*, 2865*w*, 1278*s*, 1706*s*, 1513*m*, 1503*s*, 1436*m*, 1366*s*, 1247*s*, 1222*s*, 1166*s*, 1068*m*, 932*m*, 802*s*, 772*s*, 704*s*, 694*m*. FAB-MS: 647.5 (11.0, $[2M + \text{H}]^+$), 324.2 (97.4, $[M + \text{H}]^+$), 268.2 (72.4, $[M - {}^t\text{Bu} + 2\text{ H}]^+$), 224.2 (100.0, $[M - \text{Boc} + 2\text{ H}]^+$). Anal. calc. for $C_{17}H_{25}NO_5$ (323.39): C 63.14, H 7.79, N 4.33; found: C 62.97, H 7.56, N 4.42.

Boc-β³-HSer(OBn)-β³-HSer(OBn)-OMe (**7**). The ester **6** (5.50 g, 17 mmol) was deprotected according to *GP 4* and reacted with **5** according to *GP 5*. FC (Et_2O/pentane) gave **7** (6.28 g, 72%). Colorless powder. M.p. 104–107°. R_f 0.2 (Et_2O/pentane). ^{1}H-NMR (500 MHz, $CDCl_3$): 1.42 (*s*, *t*-Bu); 2.43–2.53 (*m*, CH_2CO); 2.55–2.63 (*m*, CH_2CO); 3.45–3.49 (*m*, CH_2O); 3.53–3.61 (*m*, CH_2O); 3.62 (*s*, MeO); 4.07 (br., NCH); 4.41–4.47 (*m*, NCH); 4.49 (*s*, $PhCH_2$); 5.44 (br., NH); 6.36 (*d*, $J = 7.9$, NH); 7.27–7.36 (*m*, 10 arom. H). ^{13}C-NMR (125 MHz, $CDCl_3$): 28.61; 35.61; 37.93; 45.88; 47.70; 51.70; 70.50; 71.11; 73.23; 73.25; 127.69; 127.73; 127.76; 127.84; 128.44; 128.46; 137.77; 138.00; 155.46; 170.26; 171.83. FAB-MS: 1029.6 (9, $[2M + \text{H}]^+$); 515.3 (92, $[M + \text{H}]^+$); 415.3 (100, $[M - \text{Boc}]^+$). Anal. calc. for $C_{28}H_{38}N_2O_7$ (514.61): C 65.35, H 7.44, N 5.44; found: C 65.40, H 7.34, N 5.49.

Boc-β³-HSer(OBn)-β³-HSer(OBn)-β³-HSer(OBn)-OMe (**8**). β-Peptide **7** (5.66 g, 11 mmol) was deprotected according to *GP 4* and reacted with **5** according to *GP 5*. Recrystallization from AcOEt gave **8** (6.5 g, 84%). Colorless crystals. M.p. 140–142°. ^{1}H-NMR (300 MHz, $CDCl_3/CD_3OD$ 2:1): 1.38 (*s*, *t*-Bu); 2.37–2.39 (*m*, CH_2CO); 2.45–2.62 (*m*, 2 CH_2CO); 3.34–3.51 (*m*, 3 CH_2O); 3.57 (*s*, MeO); 4.00–4.40 (br., NCH); 4.26–4.29 (*m*, NCH); 4.37–4.41 (*m*, NCH); 4.45 (*s*, $PhCH_2$); 4.49 (*s*, 2 $PhCH_2$); 7.21–7.32 (*m*, 15 arom. H). ^{13}C- NMR (75 MHz, $CDCl_3/CD_3OD$ 2:1): 32.00; 39.69 (2 C); 41.99; 49.96; 50.74; 51.61; 55.50; 56.21; 63.96; 82.71; 83.47; 96.5; 131.61; 131.66; 132.26; 141.61; 141.66; 141.75; 159.90; 174.80; 175.19; 175.95. FAB-MS: 728.5 (34, $[M + \text{Na}]^+$); 706.5 (60, $[M + \text{H}]^+$); 606.4 (100, $[M - \text{Boc}]^+$). Anal. calc. for $C_{39}H_{51}N_3O_9$ (705.84): C 66.36, H 7.28, N 5.95; found: C 66.46, H 7.28, N 5.94.

Boc-β³-HMet-β³-HMet-OMe (**9**). Compound **4** (1.51 g, 5.44 mmol) was deprotected according to *GP 4* and reacted with **3** according to *GP 5*. Recrystallization (AcOEt) gave **9** (1.75 g, 76%). Yellowish powder. M.p. 135–

137°. $[\alpha]_D = -22.7$ ($c = 1.01$). IR ($CHCl_3$): 812*w*, 928*w*, 1052*w*, 1168*s*, 1367*m*, 1438*m*, 1497*s*, 1514*s*, 1702*s*, 2919*w*, 3008*m*, 3428*w*. ^{1}H-NMR (300 MHz, $CDCl_3$): 1.42 (*s*, *t*-Bu); 1.73–1.92 (*m*, 4 $MeSCH_2CH_2$); 2.09 (*s*, MeS); 2.09 (*s*, MeS); 2.31–2.59 (*m*, 2 $MeSCH_2$, CH_2CO); 3.69 (*s*, MeO); 3.90–3.97 (*m*, CH); 4.31–4.39 (*m*, CH); 5.37 (br. *d*, $J = 5.9$, NH); 6.36 (*d*, $J = 8.7$, NH). ^{13}C-NMR (75 MHz, $CDCl_3$): 15.57; 28.40; 30.79; 30.86; 33.32; 33.98; 38.24; 40.84; 45.45; 47.63; 51.87; 79.41; 155.60; 170.35; 171.97. FAB-MS: 423 (62, $[M+H]^+$), 323 (100, $[M+H-Boc]^+$). Anal. calc. for $C_{18}H_{34}N_2O_5S_2$ (422.61): C 51.16, H 8.11, N 6.63; found: C 51.08, H 7.96, N 6.56.

Boc-β³-HMet-β³-HMet-β³-HMet-OMe (**10**). β-Peptide **9** (1.25 g; 2.97 mmol) was deprotected according to *GP 4* and reacted with **3** according to *GP 5*. FC (CH_2Cl_2/MeOH 20:1) gave **10** (658 mg, 39%). Colorless solid. M.p. 179–182°. R_f (CH_2Cl_2/MeOH 20:1) 0.21. ^{1}H-NMR (500 MHz, $CDCl_3$): 1.44 (*s*, *t*-Bu); 1.74–1.98 (*m*, 3 $MeSCH_2CH_2$); 2.09 (*s*, MeS); 2.10 (*s*, MeS); 2.10 (*s*, MeS); 2.32–2.58 (*m*, 3 $MeSCH_2$, 3 CH_2CO); 3.70 (*s*, MeO); 3.97–4.03 (br., NCH); 4.22 (br., NCH); 4.35 (*m*, NCH); 5.33 (br., NH); 6.52 (br., NH); 6.86 (br., NH). ^{13}C-NMR (125 MHz, $CDCl_3$): 15.59; 15.61; 28.43; 30.81; 30.87; 30.98; 33.24; 33.30; 34.36; 38.34; 40.16; 41.07; 45.72; 45.91; 46.70; 47.65; 51.94; 155.75; 170.47; 170.62; 172.15. FAB-MS: 1135.5 (5, $[2M+H]^+$); 568.2 (100, $[M+H]^+$); 482.2 (62, $[M-Boc]^+$). Anal. calc. for $C_{24}H_{45}N_3O_6S_3$ (567.83): C 50.77, H 7.99, N 7.40; found: C 50.95, H 7.82, N 7.46.

Cyclo(β³-HSer(OBn)-β³-HSer(OBn)-β³-HSer(OBn)) (**11**). β-Peptide **8** (4.94 g, 7.0 mmol) was dissolved in TFE (60 ml). NaOH (8.4 g, 210 mmol) in H_2O (42 ml) was added, and the resulting mixture was stirred for 1 d at r.t. and for 6 h at 40°. The pH value of the resulting soln. was adjusted to 2–3 with 1M HCl soln., and the soln. was extracted with AcOEt (3×). The org. layer was dried ($MgSO_4$) and evaporated. The resulting Boc-β-peptide acid (4.0 g; 5.8 mmol) was dissolved in DMF (300 ml) and cooled under Ar to 0°. Pentafluorophenol (1.29 g, 7 mmol) and EDC (1.34 g, 7.0 mmol) were added, and the resulting soln. was stirred at r.t. overnight. The solvent was evaporated, and the residue was taken up in $CHCl_3$ and washed with 1M HCl and sat. NaCl solns. The solvent was removed under reduced pressure and dried under h.v. The resulting β-peptide ester was deprotected according to *GP 4*, dissolved in 280 ml of MeCN (0.025M) and added by syringe pump over 7 h to a soln. of $EtN(i\text{-}Pr)_2$ (1.36 g, 10.5 mmol) in MeCN (1700 ml, 3.3 mM) at 70°. The suspension was stirred at r.t. overnight. The resulting precipitate was filtered and purified by carefully washing with 0.5M HCl, 0.5M NaOH, sat. NaCl solns., and MeCN to give **11** (810 mg, 20% based on **8**). Colorless powder. M.p. >215°. IR (KBr): 3286*s*, 3088*w*, 3031*w*, 2858*m*, 2794*w*, 1652*s*, 1559*s*, 1452*m*, 1364*m*, 1314*w*, 1271*w*, 1198*m*, 1130*s*, 1099*s*, 1027*m*, 734*m*, 696*s*, 668*m*, 609*w*, 527*w*, 466*w*. ^{1}H-NMR (400 MHz, $CDCl_3$/10 drops CF_3CO_2D): 2.54–2.64 (*m*, 3 CH_2CO); 3.53–3.64 (*m*, 3 $BnOCH_2$); 4.42–4.46 (br., 3 NCH); 7.25–7.38 (*m*, 15 arom. H). ^{13}C-NMR: 32.55; 37.78; 48.44; 69.96; 73.58; 109.21; 128.22; 128.62; 128.77; 136.07; 173.26. FAB-MS: 1185.6 (15, $[2M+K]^+$), 1167.6 (6, $[2M+Na]^+$), 1147.7 (7, $[2M+H]^+$), 612.3 (6, $[M+K]^+$), 596.3 (57, $[M+Na]^+$), 574.3 (100, $[M+H]^+$).

Cyclo(β³-HMet-β³-HMet-β³-HMet) (**12**). Compound **10** (503 mg, 0.89 mmol) was dissolved in TFE (7.1 ml, 0.03M soln.). NaOH (1.77 g, 44.3 mmol) in H_2O (8.9 ml) were added, and the resulting mixture was stirred for 2 d. The pH value of the resulting soln. was adjusted to 2–3 with 1M HCl soln. and extracted with AcOEt. The org. layer was dried ($MgSO_4$) and evaporated. The resulting Boc-β-peptide acid (475 mg, 0.86 mmol) was dissolved in DMF (43 ml; 0.02M) and cooled under Ar to 0°. Pentafluorophenol (171 mg, 0.93 mmol) and EDC (172 mg, 0.90 mmol) were added, and the resulting soln. was stirred at r.t. for 8 h. The solvent was evaporated, the residue was taken up in $CHCl_3$ and washed with 1M HCl and sat. NaCl solns. The solvent was removed under reduced pressure and dried under h.v. The resulting β-peptide ester (555 mg, 0.77 mmol) was deprotected according to *GP 4* and dissolved in MeCN (31 ml, 0.025M). This soln. was added by syringe pump over 4 h to a soln. of $EtN(i\text{-}Pr)_2$ (0.24 ml, 1.39 mmol) in MeCN (420 ml, 3.3mM) at 70°. The resulting precipitate was filtered and purified by washing with 0.5M HCl, 0.5M NaOH, and sat. NaCl solns. to give **12** (125 mg, 32% based on **10**). Off-white solid. M.p. >215°. IR (KBr): 3281*s*, 3079*w*, 2913*m*, 2850*w*, 1653*s*, 1559*s*, 1438*m*, 1368*w*, 1294*w*, 1207*w*, 1145*w*, 951*w*, 695*w*, 632*w*. ^{1}H-NMR (300 MHz, $CDCl_3$/10 drops TFA): 1.84–1.92 (*m*, 3 $MeSCH_2CH_2$); 2.11 (*s*, 3 MeS); 2.46–2.61 (*m*, 3 CH_2CO); 2.81 (*m*, 3 $MeSCH_2$); 4.48 (br., 3 CH); 7.54 (br., 3 NH). ^{13}C-NMR (75 MHz, $CDCl_3$/10 drops TFA): 14.91; 29.97; 32.85; 40.74; 48.51; 173.91. FAB-MS: 909.3 (9, $[2M+K]^+$), 871.4 (6, $[2M+H]^+$), 498.1 (17.3), 458.2 (17 $[M+Na]^+$), 436.2 (100, $[M+H]^+$).

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