Substitution of Amino Acids 1 and 3 in Teicoplanin Aglycon: Synthesis and Antibacterial Activity of Three First Non-natural Dalbaheptides

Adriano Malabarba, Romeo Ciabatti, Erminio Gerli, Franca Ripamonti,

Pietro Ferrari and Luigi Colombo

Lepetit Research Center,
Via R. Lepetit 34, 21040 Gerenzano (Varese), Italy

Eugenia N. Olsufyeva, Andrey Y. Pavlov, Marina I. Reznikova,

Eduard I. Lazhko and Maria N. Preobrazhenskaya

Institute of New Antibiotics, Russian Academy of Medical Sciences,
B. Pirogovskaya 11, Moscow 119867, Russia

The replacement of amino acids 1 and 3 of glycopeptide antibiotics (dalbaheptides) with new amino acids or other chemical entities suitable to interact with both glycopeptide-resistant (D-Ala-D-Lactate) and susceptible (D-Ala-D-Ala) targets is one of the chemical strategies currently followed to pursue activity against highly glycopeptide-resistant VanA enterococci while maintaining activity against glycopeptide-susceptible Gram-positive bacteria, particularly methicillin-resistant staphylococci. As a preliminary approach, the substitution of amino acid 1 of deglucoteicoplanin (TD) with D-lysine or D-methylleucine and of its amino acid 3 with L-phenylalanine or L-lysine was investigated.

In this paper, the synthesis and *in vitro* antibacterial activities of first non-natural dalbaheptide methyl ester aglycons MDL 63,166 (D-Lys^1-Phe^3-TD-DHP-Me), MDL 64,945 (D-Lys^1-Lys^3-TD-DHP-Me), and MDL 64,468 (D-MeLeu^1-Lys^3-TD-DHP-Me) are described. These compounds, which were obtained from intermediate TD-derived tetrapeptide methyl ester (TDTP-Me) according to a 9-step overall procedure, had excellent anti-staphylococcal activity. The most active derivative against staphylococci, MDL 64,945 (MIC: 0.063 μg/ml for *S. aureus*, *S. epidermidis* and *S. haemolyticus*) was inactive against VanA enterococci, while MDL 63,166 and MDL 64,468 were somewhat active against VanA strains of *E. faecalis*; MDL 64,468 was also moderately active against one VanA isolate of *E. faecium* and had marginal activity as TD against *E. coli*.

The increased interest for glycopeptides of the dalbaheptide family[1] is related to the increasing incidence of serious and often multi-resistant Gram-positive pathogens infecting hospital patients, such as the difficult-to-treat methicillin-resistant (Meth-R) *Staphylococcus aureus* (MRSA), as virulent pathogen of major importance, and coagulase-negative staphylococci (CNSt)[†], as the most frequent cause of bacteremia particularly in immunocompromised patients. Currently commercially available glycopeptides vancomycin (V)[2] and teicoplanin (T)[3] are extensively used in the treatment of severe infections caused by MRSA and CNSt as well as in the therapy of enterococcal infections. Infections due to enterococci have become a dramatic clinical problem since few antibacterial agents are efficacious against these refractory organisms and increasing resistance is rapidly eliminating the current

options. The recent emergence and spread of resistance also to glycopeptides in VanA enterococci poses a serious threat for the future.[4] Even some strains of Meth-R CNSt have reduced susceptibility to T and occasionally to V.[5] It follows an urgent need for new more potent glycopeptides against multi-resistant Gram-positive pathogens which combine improved activity against Meth-R staphylococci with excellent activity against highly glycopeptide-resistant VanA enterococci.

Resistance to glycopeptides in VanA enterococci is due to a change in the structure of target peptide D-Ala-D-Ala which is replaced by a D-Ala-D-Lactate depsipeptide[6] that is not sufficiently bound by glycopeptides to prevent the bacterial growth. In the past decade, structure-activity relationships (SAR) established with chemical derivatives of V or lipoglycopeptides T and A-40,926 (A-40)[7] clearly indicated that by chemical derivatization there is the

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[†] Most of CNSt are Meth-R strains.

possibility to improve the activity against Gram-positive pathogens when the target peptide D-Ala-D-Ala is unmodified. So far, none of derivatives of either V, T or A-40 has shown improved affinity for glycopeptide-resistant target D-Ala-D-Lactate. The moderate activity against VanA enterococci shown by some acetylglucos-amine-less teicoplanin-type derivatives[8,9] might be ascribed to a modified mechanism likely based on lipophilic side-interactions at the level of the cytoplasmic membrane of these bacteria, as proposed for a class of derivatives of chloroeremomycin (Eli Lilly)[10] recently claimed to possess potent activity against V-resistant enterococci, including VanA phenotypes. Since it seems that all glycopeptide derivatives somewhat active against VanA enterococci easily dimerize, it was hypothesized that dimerization might improve affinity for peptidogly-can precursors by simultaneous interaction with the terminal chains of growing peptidoglycan and a next disaccharide intermediate. This intramolecular effect, which would be favored by the presence of lipophilic side chains able to anchor the glycopeptide molecules to the membrane, would prevent further transglycosylation reaction. However, non-specific lipophilic interactions with the membrane cannot be excluded in principle.

The current objective of chemical programs of trans-formation of glycopeptides is to achieve simultaneous interaction with both susceptible and resistant targets. This could be likely pursued by changing their binding properties by suitable modifications of the structure of the heptapeptide backbone in the active site region. An inkling of one of these possibilities has been obtained with the discovery of glycopeptide-derived tetrapep-tides[11] which are the most useful synthons for the introduction of new chemical entities in positions 1 and 3.

The present paper demonstrates the chemical feasi-bility of this approach by the synthesis of three non-natural dalbaheptides from deglucoteicoplanin(TD)-derived tetrapeptide (TDTP) suitably protected at one carboxy group as methyl ester (TDTP-Me) and at one amino group as benzyl carbamate (N^2-CBZ-TDTP-Me, Fig. 1).[11] In particular, the synthesis and some antibacterial properties of D-Lys[1]-Phe[3] (MDL 63,166: 7a), D-Lys[1]-Lys[3] (MDL 64,945: 7b), and D-MeLeu[1]-Lys[3] (MDL 64,468: 7c) methyl ester dalbaheptides (Fig. 2) deriving from TD are described. The choice of the D-MeLeu as new amino acid 1 was based on the expected ability of this amino acid to enhance binding to target peptides.[12] The replacement of D-MeLeu with D-Lys was also suggested by recent findings that dalbaheptides terminating with one of these amino acids have similar antibacterial properties.[13] Preliminary attempts to synthesize a further dalbaheptide containing L-histidine in position 3 are also reported.

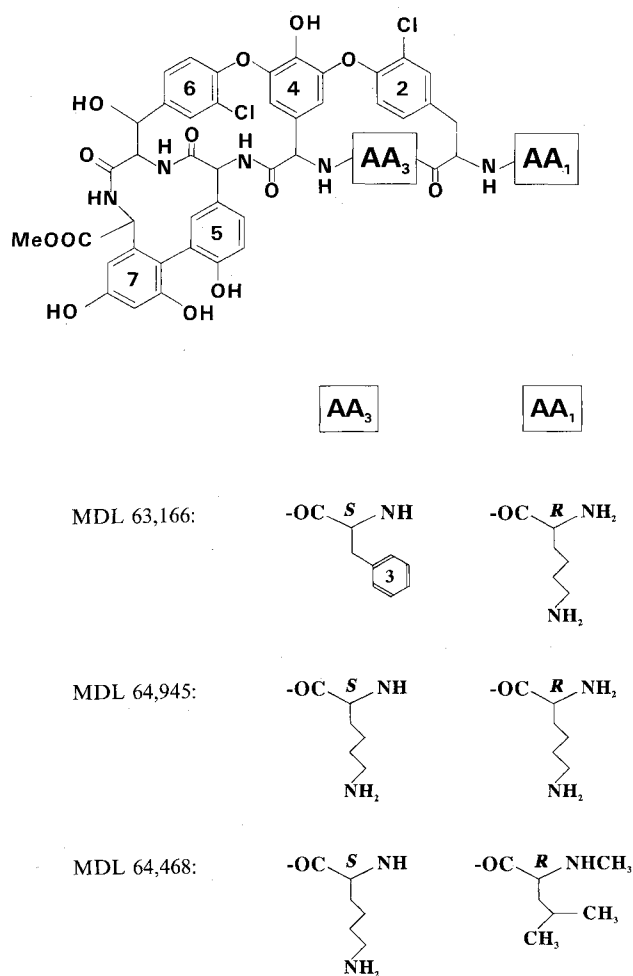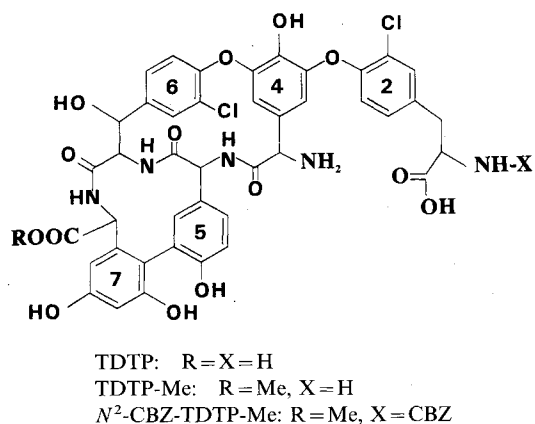Fig. 2. Structure of MDL 63,166, MDL 64,945 and MDL 64,468.

MDL 63,166:	-OC S NH (3)	-OC R NH₂
MDL 64,945:	-OC S NH	-OC R NH₂
MDL 64,468:	-OC S NH	-OC R NHCH₃

Fig. 1. Structure of TDTP, TDTP-Me, and N^2-CBZ-TDTP-Me.

TDTP: R = X = H
TDTP-Me: R = Me, X = H
N^2-CBZ-TDTP-Me: R = Me, X = CBZ

Chemistry

The three first non-natural dalbaheptides **7a**, **7b** and **7c** were prepared from N^2-CBZ-TDTP-Me (**1**) according to a six-step[††] procedure (Schemes 1~5) in 8, 5, and 2.4% overall yields, respectively. For the synthesis of compounds **7a** or **7b**, interaction of the free amino group of **1** with N-BOC-phenylalanine, N-hydroxysuccinimide (NSu) ester or N_α-FMOC-N_ϵ-BOC-L-lysine, pentafluorophenyl (Pf) ester produced N-BOC-Phe- (**2a**) or N_α-FMOC-N_ϵ-BOC-L-Lys (**2b**) containing pentapeptides, respectively. Deprotection of **2a** with TFA gave **3a**, while deprotection of the N_α-amino group of the lysyl moiety of compound **2b** with piperidine yielded **3b**. The most challenging step was macrocyclization of **3a** and **3b** to hexapeptides **4a** and **4b**, respectively, which was carried out in the presence of hydroxybenzotriazole (HOBT), N-methylmorpholine and DCC. Hydrogenolysis (H$_2$, 5% Pd/C) of the N-terminal CBZ group of **4a** and **4b** gave hexapeptides **5a** and **5b**, respectively. Reaction of **5a** or **5b** with di-Boc-D-Lys, NSu ester, produced (di-BOC-D-Lys)^1-(Phe)^3-TD-Me (**6a**) or (di-BOC-D-Lys)^1-(N_ϵ-BOC-L-Lys)^3-TD-Me (**6b**); final removal of the BOC protective groups (TFA) yielded **7a** or **7b**. The heptapeptide (N_α-

BOC-N-Me-D-Leu)^1-(N_ϵ-BOC-Lys)^3-TD-Me (**6c**) was synthesized by reaction of **5b** with N-BOC-D-methylleucine, HOBT ester (prepared *in situ*). The N-BOC-D-methylleucine was prepared (79%) from N-BOC-D-leucine as previously described.[14] The HOBT ester of N-BOC-D-MeLeu was obtained from N-BOC-D-MeLeu and HOBT in the presence of DCC. Deblocking of **6c** (TFA) gave **7c**.
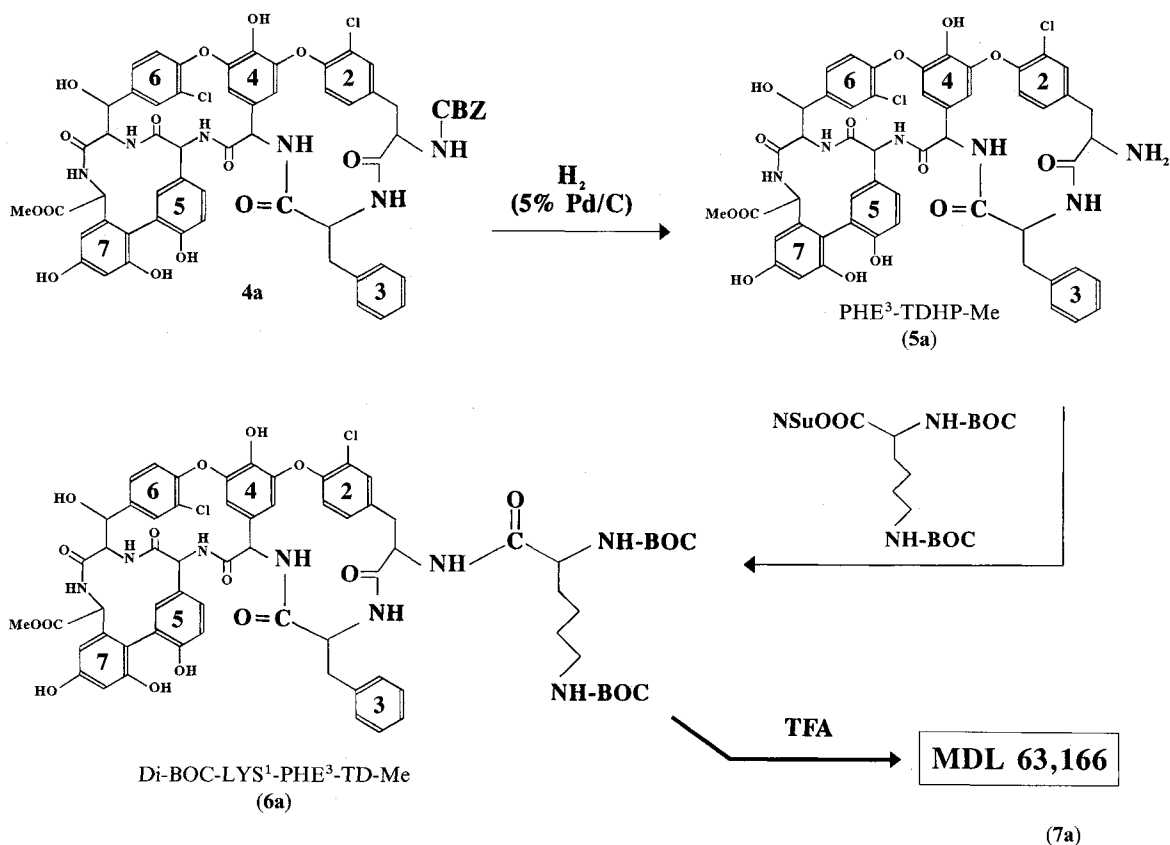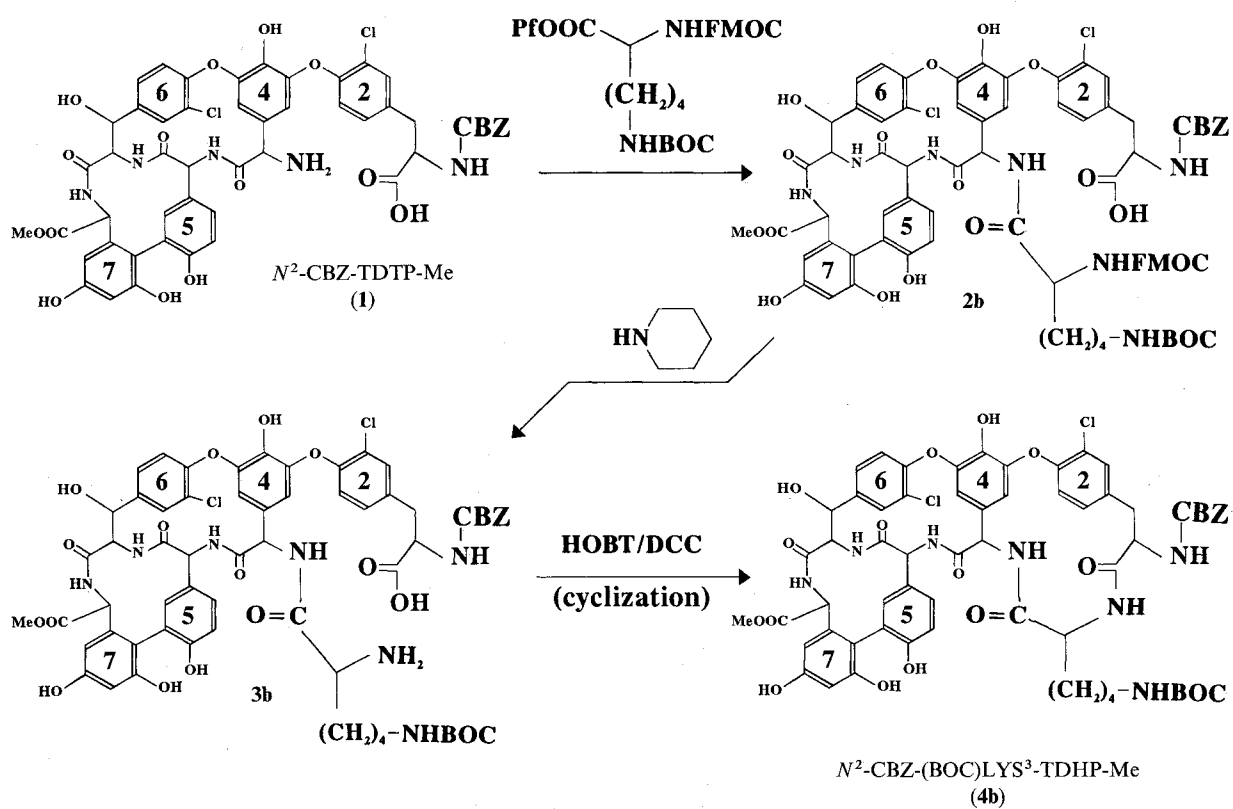
Interaction of N_α-CBZ-N_{im}-BOM-histidine, Pf ester with the free amino group of **1** in DMF (Scheme 6), yielded N^2-CBZ-(N_α-CBZ-N_{im}-BOM-His)^3-pentapeptide, methyl ester (**2d**). Selective removal (TFA) of the BOC group gave N^2-CBZ-(N_{im}-BOM-His)^3-pentapeptide methyl ester (**3d**) which was submitted to the macrocyclization step obtaining N^2-CBZ-(N_{im}-BOM-His)^3-hexapeptide, methyl ester (**4d**; 1.8% overall yield from **1**). This is a suitable intermediate in the synthesis of (His)^3-containing dalbaheptides. If these compounds will be of interest, further work is needed to increase their preparation yields. So far, attempts to synthesize a histidine-containing hexapeptide without protection of the imidazole ring failed.
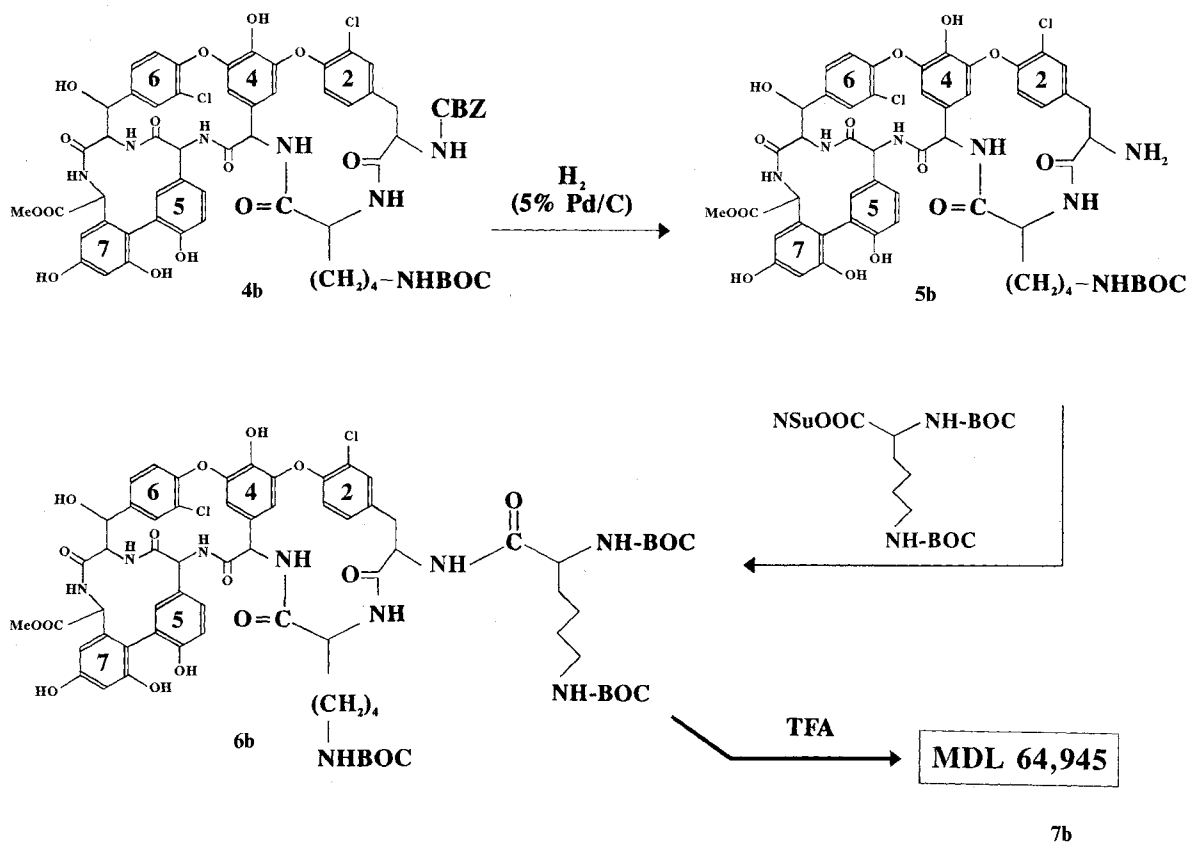
Some analytical data of final compounds are given in

Scheme 1.   Introduction of AA$_3$ (L-Phe) and cyclization.

N^2-CBZ-TDTP-Me
(**1**)

N^2-CBZ-PHE^3-TDHP-Me
(**4a**)
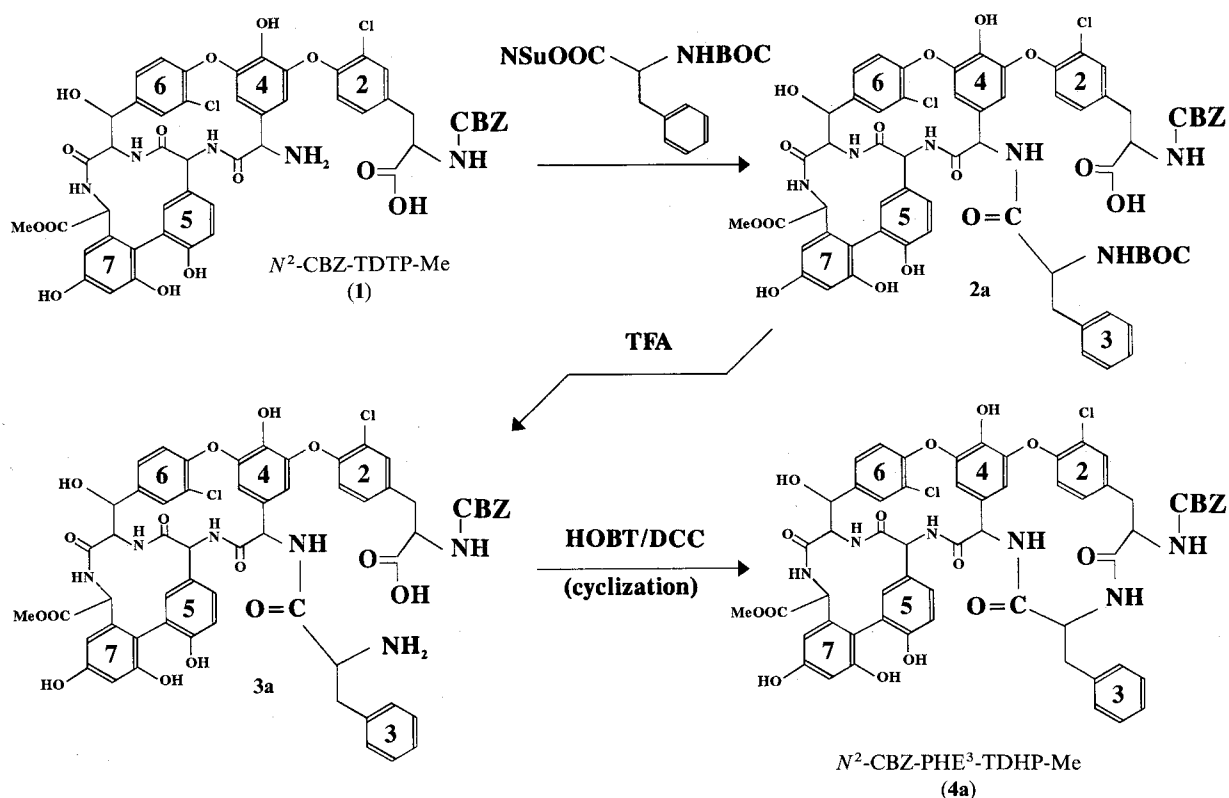
[††]   Nine steps from unprotected TDTP-Me.

Scheme 2. Introduction of AA$_1$ (D-Lys).

PHE^3-TDHP-Me
(5a)

Di-BOC-LYS^1-PHE^3-TD-Me
(6a)

MDL 63,166

(7a)

Scheme 3. Introduction of AA$_3$(L-Lys) and cyclization.

N^2-CBZ-TDTP-Me
(1)

2b

3b

N^2-CBZ-(BOC)LYS^3-TDHP-Me
(4b)

Scheme 4. Introduction of AA$_1$ (D-Lys).

Scheme 5. Introduction of AA$_1$ (D-MeLeu).
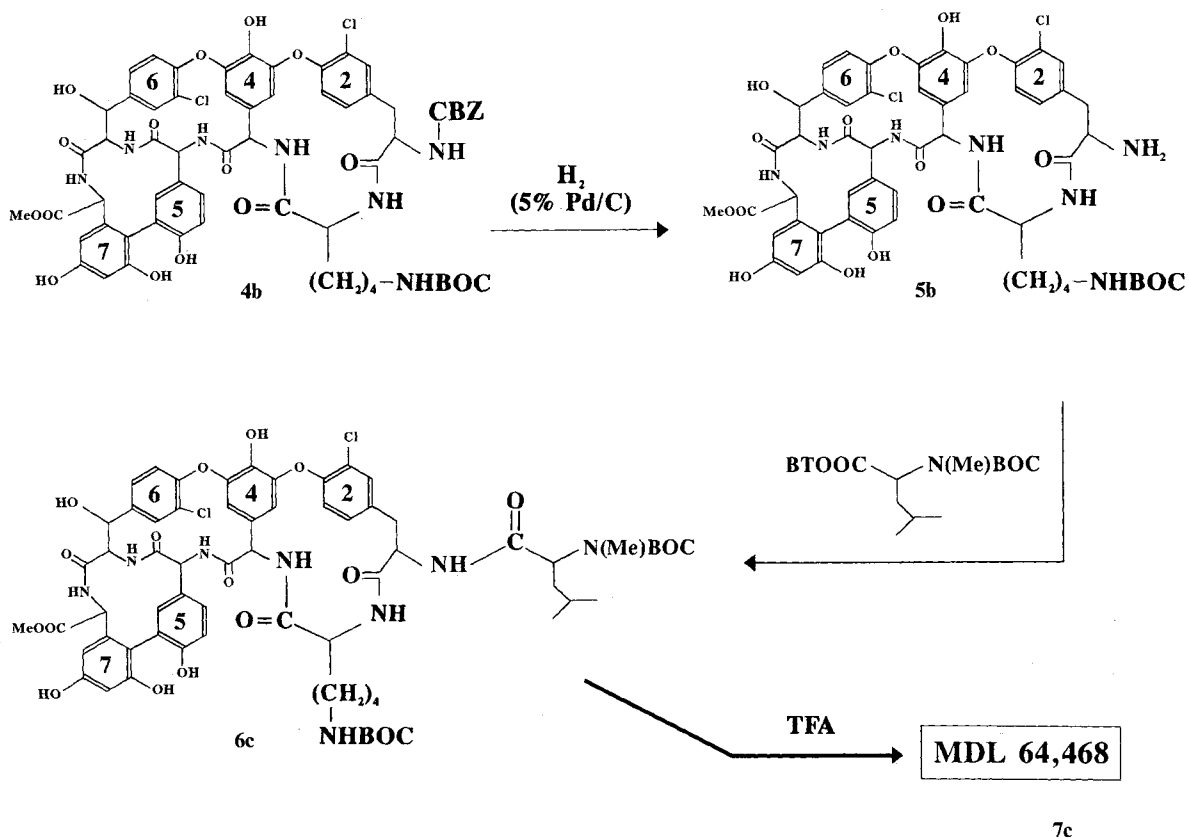
Scheme 6. Introduction of AA$_3$ (L-His) and cyclization.

N^2-CBZ-TDTP-Me
(1)

2d

TFA

HOBT/DCC
(cyclization)

3d

N^2-CBZ-(BOM)His^3-TDHP-Me
(4d)
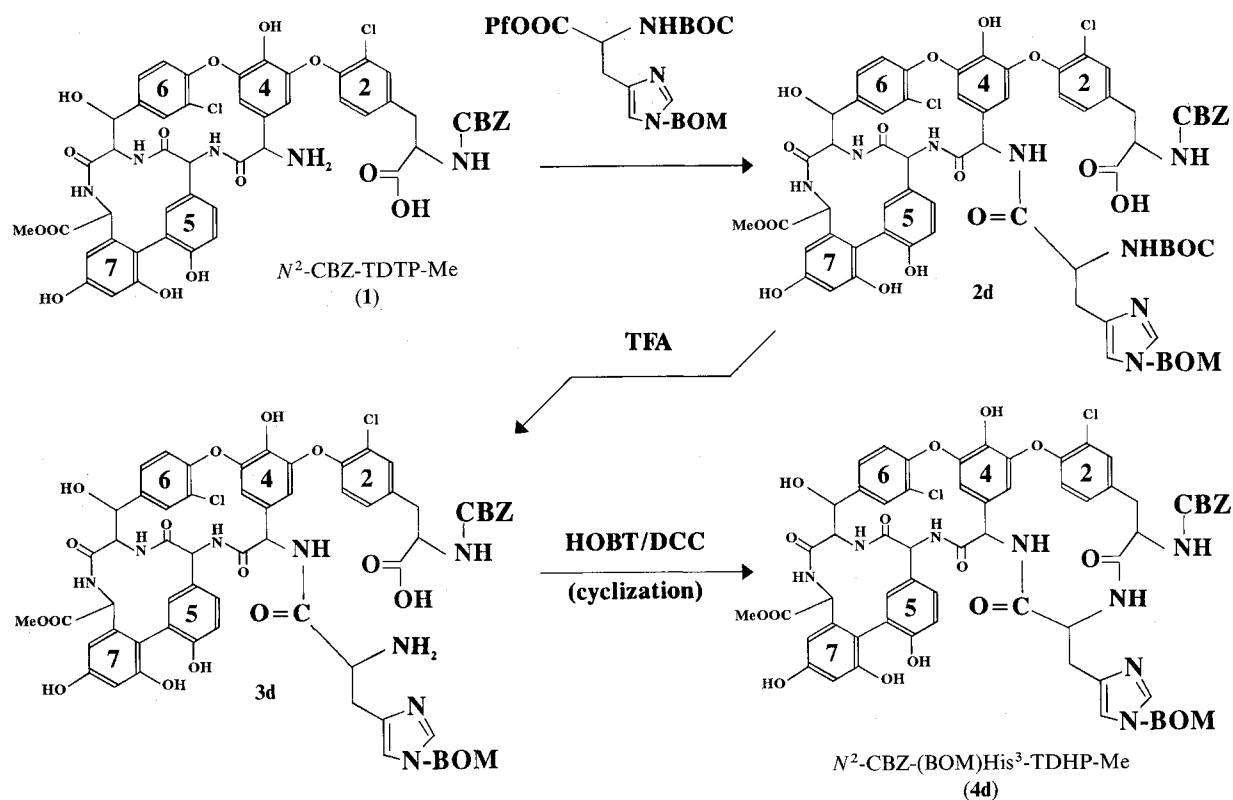
Table 1.

Compound	Yield[a] (%)	HPLC[b] (Rt, minutes)	ESI-MS[c] [M+H]$^+$	MW[d]	Formula
MDL 63,166	8	18.3	1175.3	1176.03	$C_{58}H_{56}N_8O_{15}Cl_2$
MDL 64,945	5	8.3	1156.4	1157.04	$C_{55}H_{59}N_9O_{15}Cl_2$
MDL 64,468	2.4	8.6	1155.4	1156.05	$C_{56}H_{60}N_8O_{15}Cl_2$

[a] From N^2-CBZ-TDTP-Me (1). [b] See Experimental Section. [c] Monoisotopic mass. [d] Average mass.

Table 1.

Structure Elucidation

The structures of intermediates N-CBZ-protected hexapeptide **4a**, CBZ, BOC-diprotected hexapeptide **4b**, di-BOC-protected heptapeptide **6c** (Tables 2 and 3), and those of MDL 63,166, MDL 64,945 and MDL 64,468 (Table 4) were determined by ^{1}H NMR and confirmed by electrospray ionization mass spectrometry (ESI-MS).

The ^{1}H NMR spectra attributions (Tables 4 and 5) were based on COSY proton-proton correlations and on comparison with data reported for TD.[15] Assignments concern amino acid residues 1 and 3 which replace those of TD methyl ester (TD-Me) in these positions. The reported chemical shift values are not significantly different from those expected, except for the α-CH$_2$ protons of the Lys moiety whose chemical shift value is at very high field. This can be interpreted as due to the shielding effect exerted on this CH$_2$ by the amide carbonyl group of Lys itself in the peptide chain.

In Table 5, the ^{1}H NMR assignments for some selected protons in the left-hand part of the molecules of these new dalbaheptides are given in comparison with those of corresponding TD-protons. The similarities with the latter compound suggest that the stereochemistry and the 3D structure in the left-hand part of these molecules are unchanged. The strong change in the chemical shift of proton 4b suggests that along with chemical modification this part of the molecule also experiences a 3D change.

Table 2. ^{1}H NMR data (DMSO-d_6) for N^2-CBZ-hexa-peptide **4a**.

Protons[a]	Delta (ppm)
x2	4.60
x3	4.25
x4	5.82
x5	4.60
x6	4.25
x7	4.54
z2, z2'	2.82, 2.72
Phe-CH$_2$	2.43, 2.25
z6	5.13
CBZ-CH$_2$	4.97
4b	6.23
4f	5.40
w2	7.45
w3	7.70
w4	8.38
w5	8.69
w6	6.60
w7	8.65
COO-CH$_3$	3.71
6b	7.87
7d	6.44
7f	6.10

[a] The proton nomenclature is that adopted for TD.[15] By analogy with the proton nomenclature adopted for gly-copeptides, HCα protons of amino acid fragments (n = 1 to 7) are indicated with symbol "x$_n$", CH(OH) or CH$_2$ benzyl protons are defined as z$_n$ or z$_n$, z$_n'$, while symbol w$_n$ is used for NH or NH$_2$ protons. Aromatic, benzylic and HCα protons are defined as described in ref. 15.

Table 4. Significant ^{1}H NMR data (delta, ppm; DMSO-d_6, 303 °K) of MDL derivatives.

Protons	MDL 63,246	MDL 64,945	MDL 64,468
AA1-αCH	3.65	3.70	3.72
AA3-αCH	4.12	4.36	4.36
AA4-αCH	5.90	5.79	5.82
AA2-CH$_2$	3.22, 2.88	3.02, 2.95	3.15, 2.74
Phe-CH$_2$	3.50~3.30	—	—
AA1-CH$_2$'s	2.72 (5), 1.50 (4), 1.35 (3), 1.72 (2)	2.72 (5), 1.50 (4), 1.35 (3), 1.75 (2)	1.55
AA3-CH$_2$'s	—	2.72 (5), 1.48 (4), 1.09 (3), 0.77 (2)	2.70 (5), 1.48 (4), 1.09 (3), 0.77 (2)
COO-CH$_3$	3.71	3.71	3.70

Antibacterial Activity

The antibacterial activity of the three new dalbahep-tides (Table 6) was particularly interesting, considering that MDL 63,166 and MDL 64,468 had moderate ac-tivity against VanA enterococci while all reference

Table 3. ^{1}H NMR data of the compounds **4b**[a] and **6c**.[b]

Proton	4b δ (ppm)	6c δ (ppm)	Proton	4b δ (ppm)	6c δ (ppm)
x2	4.40	4.80	2b	7.38	7.38
2e	7.20	7.02	2e	7.20	7.02
x3	4.30	4.29	2f	7.45	7.17
x4	5.73	5.70	4b	6.07	5.80
x5	4.58	4.56	4f	5.34	5.16
x6	4.24	4.23	5b	7.23	7.22
x7	4.55	4.50	5e	6.83	6.69
w2	[c]	[c]	5f	6.72	6.79
w3	7.40	7.30	6b	7.85	7.84
w4	8.15	8.33	6e	7.10	7.21
w5	8.59	8.78	6f	6.95	7.44
w6	6.49	6.62	7d	6.41	6.41
w7	8.59	8.66	7f	6.12	6.07
z2	3.02	3.01	OMe	3.71	3.69
z'2	2.81	2.87	α-CH$_2$	0.82	0.60
z6	5.13	5.03	β-CH$_2$	1.30	1.30
OH-z6	5.84		γ-CH$_2$	1.30	1.30
δ-NH	[c]	6.64	δ-CH$_2$	2.81	2.87, 2.80
t-Bu	1.36	1.38			

[a] Signals of OCH$_2$-group are observed at 5.01 ppm, Ph-group—as broad singlet at 7.28 ppm.

[b] Signals of N-BOC-D-Me-D-Leu: 4.80 (α-CH); 2.43 (N-CH$_3$); 1.50 and 1.40 (CH$_2$); 1.40 (CH of iso-propyl); 1.38 (t-Bu); 0.86 and 0.84 (CH$_3$ of iso-propyl).

[c] Signals were not observed.

Table 5. Comparison of selected ^{1}H NMR data for the three dalbaheptides and TD.

(delta, ppm)

Proton	TD	MDL 63,166	MDL 64,945	MDL 64,468
x4	5.60	5.90	5.79	5.82
x5	4.33	4.53	4.52	4.50
x6	4.10	4.12	4.23	4.20
x7	4.42	4.57	4.50	4.20
4b	5.50	5.97	5.97	5.87
4f	5.08	5.27	5.27	5.26
6b	7.77	7.78	7.88	7.86
z6	5.10	5.09	5.12	5.11

glycopeptides, including the methyl ester (TD-Me) of TD[†††], were devoid of activity against these bacteria.

The inactivity of MDL 64,945 against Gram-negative *E. coli* was unexpected since the two lysines in positions 1 and 3 were thought to promote penetration through the Gram-negative outer membrane according to a mechanism of self-promoted uptake.[16]

However, a common property of these three com-

[†††] In a separate experiment, also the methyl ester of the aglycon of V-type glycopeptide eremomycin[1] (a sample of this compound was supplied from the Inst. New Antibiotics, Moscow) was inactive against these strains of VanA enterococci; unpublished results, Lepetit Research Center laboratories.

Table 6. *In vitro* activity in comparison with TD, its methyl ester (TD-Me), teicoplanin and vancomycin.

| | | | | | | MIC (μg/ml) |
| | Staphylococcus aureus Tour | Staphylococcus epidermidis ATCC 12228 | Staphylococcus haemolyticus L 602 | Streptococcus pyogenes C 203 | Enterococcus faecalis ATCC 7080 | Enterococcus faecalis L 562 (VanA) |
Compound						
MDL 63,166	0.125	0.125	0.5	0.25	0.5	16
MDL 64,945	0.063	0.063	0.063	0.5	0.5	>128
MDL 64,468[a,b]	0.25	0.125	0.125	0.125	N.D.	32
TD[b]	0.125	0.063	0.25	0.125	0.125	>128
TD-Me[b]	0.125	0.125	0.25	0.125	0.25	>128
Teicoplanin	0.125	0.25	8	0.125	0.125	>128
Vancomycin	0.25	0.125	1	0.25	0.5	>128

N.D.: Not determined.

[a] Additional MIC values for MDL 64,468: 0.125 and 64 μg/ml against *E. faecalis* L 559 and L 560 (VanA, isogenic of L 559), respectively; 0.25 and 16 μg/ml against *E. faecium* L 568 and L 569 (VanA, isogenic of L 568), respectively.

[b] Against *E. coli* SKF 12140, MDL 64,468 and TD: MIC 64 μg/ml; TD-Me: MIC 16 μg/ml; all the other compounds: MIC >128 μg/ml.

pounds was their excellent activity against other Gram-positive isolates. Of particular interest was the *in vitro* anti-staphylococcal activity of MDL 64,945 which proved to be significantly superior to reference dalbaheptides against *S. haemolyticus*.

These results clearly indicate that enhanced activity against glycopeptide-resistant enterococci and other Gram-positive pathogens could be pursued by replacement of amino acids 1 and 3 in the heptapeptide structure and also confirm the importance of the structure of these amino acids for the activity of dalbaheptides.

Experimental

HPLC Methods

Reactions, column eluates and final products were checked by HPLC.

For MDL 63,166, and corresponding intermediate compounds (a series), HPLC analyses were performed (Method A) on a column LiChroCART (125 × 4 mm, Merck) pre-packed with LiChrospher RP-8 (5 μm), using a Varian Model 5500 Liquid Chromatographic pump equipped with a 20 μl loop injector Rheodyne Model 7125 and a UV variable detector. Chromatograms were recorded at 254 nm. Elutions were carried out at a flow-rate of 1.5 ml/minute by mixing eluent a, 0.2% aqueous ammonium formate, with eluent b, acetonitrile, according to a linear gradient programmed as follows:

Time (minutes): 0 30 35 40 45
% of b in a: 20 60 75 75 20

For the other compounds (b, c and d series) HPLC analyses were performed on a Partisil ODS column (250 mm × 4.6 mm i.d., particle size 10 μm, Whatman; injection volume 5 μl) using a Shimadzu liquid chromatograph of LC-10 series. All chromatogramms were

Acetonitrile-buffer linear gradient

System	Linear gradient (% of acetonitrile)	Minute	Buffer	Flow rate (ml/ minute)
B	2→24	20	H₃PO₄, 0.01 M pH 2.6	2.0
C	30→75	30	HCOONH₄ 0.2% pH 6.4	2.0
D	20→80	40	HCOONH₄ 0.2% pH 3.8	1.5

recorded at 280 nm by UV variable detector. These compounds were purified using a HPLC semipreparative column Zorbax ODS (250 mm × 9.4 mm i.d.; particle size 10 μm, injection volume 500 μl). The elution systems used were as follows:

¹H NMR

The ¹H NMR spectra were recorded in DMSO-d_6 solution at 303°K on a Bruker AM 500 NMR-spectrometer equipped with an Aspect 3000 computer, using Me₄Si (delta, 0.00 ppm) as internal reference.

Signal assignments in the ¹H NMR spectra of compounds **4b** and **6c** were accomplished using DQCOSY and ROESY experiments. In these cases, NMR spectra were recorded on a Varian VXR-400 spectrometer at 323°K. From the DQCOSY spectra were identified three ABX-spin systems which belong to the aromatic rings 2, 5, 6 and two AX-spin systems which belong to the protons of the aromatic rings 4 and 7. The signals of x2, x3, x4, x5, x6, x7 protons were detected from ROESY experiments. The combination of DQCOSY and ROESY data allowed the assignment of all remaining protons.

ESI-MS

An API III + triple quadrupole mass spectrometer (PE-Sciex, Thornhill, ON, Canada) equipped with an articulated ion spray interface was employed. Tuning and calibration were performed on both the first (Q1) and the third quadrupole using a solution of polypropylene glycols (PPG's) in 3 mM ammonium acetate. Samples were infused via a 75 μm i.d. fused silica capillary tubing to the ion spray tip which was held at a potential of 5.5 kV. A syringe pump (Model 22, Harvard Apparatus, MA, U.S.A.) controlled the delivery of the sample at a rate of 5.0 μl/minute. Zero grade compressed air was used as nebulizer gas (pressure set at 55 psi). A curtain gas (99.999% UHP nitrogen) flow of 0.8 liter/minute was employed. The interface heater was set at 60°C. Mass spectra were obtained at a dwell time of 1.00 msec (Q1 scan range 300 ÷ 1800 u, 10 scans averaged) and a step size of 0.10 u. The orifice voltage was maintained at 80 V. All samples (ca. 100 μg/ml) were dissolved, immediately prior to analysis, in a 0.1% aqueous TFA - MeOH 1 : 1 mixture.

Elemental Analysis

Because of the minimum amount of final compounds, only inorganic residue (combustion at 900°C in O_2 atmosphere: <0.2% for all final derivatives) and solvent content (TGA: ca. 10%, mainly water, for all final compounds) were determined. The identity and purity of the MDL derivatives was established by NMR, ESI-MS, and HPLC analyses.

Preparation of MDL 63,166

N^2-CBZ-N^3-BOC-Phenylalanyl-TDPP (TD-Penta-Peptide), Methyl Ester (2a)

To a stirred solution of 1 g (about 0.9 mmol) of compound 1 in 20 ml of DMF, 0.13 ml (about 0.9 mmol) of TEA was added at room temperature followed by 0.34 g (about 0.9 mmol) of N-BOC-L-Phenylalanine-N-hydroxy-succinimide ester. After 3 hour stirring at room temperature, 100 ml of water was added and the resulting solution was adjusted at pH 3 with 1 N HCl. Extraction with n-BuOH (100 ml) and evaporation of the solvent yielded 0.9 g of the title compound (HPLC: Method A, Rt 14.9 minutes).

$C_{65}H_{60}N_6O_{19}Cl_2$, MW[††††] 1300.1; ESI-MS[†††††]: m/z 1299.3, [M + H]$^+$

N^2-CBZ-Phe^3-TDPP, Methyl Ester (3a)

A solution of 220 mg of the above compound (2a) in 10 ml of dry TFA was stirred at room temperature for 15 minutes, and then the solvent was evaporated at 30°C under reduced pressure. The oily residue was slurried with Et$_2$O to give 200 mg of the title compound, as the trifluoroacetate (HPLC: Method A, Rt 10.2 minutes).

$C_{60}H_{52}N_6O_{17}Cl_2$, MW 1200.0; ESI-MS: m/z 1199.3,

[M + H]$^+$

N^2-CBZ-Phe^3-TDHP (TD-HexaPeptide), Methyl Ester (4a)

To a stirred solution of 0.4 g (about 0.34 mmol) of compound 3a in 40 ml of a DMF - CH$_2$Cl$_2$ 1 : 1 mixture, 0.047 g (about 0.34 mmol) of hydroxybenzotriazole (HOBT) hydrate and 0.038 ml (about 0.34 mmol) of N-methyl-morpholine were added at room temperature followed by 0.085 g (about 0.4 mmol) of dicyclohexyl-carbodiimide (DCC). The reaction mixture was stirred at room temperature overnight, and then the chlorinated solvent was evaporated at 30°C under reduced pressure. Afterwards, 200 ml of a H$_2$O - EtOAc 1 : 1 mixture was added dropwise under stirring. The resulting mixture was adjusted at pH 3 with 1 N HCl and the insoluble matter was filtered off. Then the organic layer was separated and the solvent evaporated at 35°C under reduced pressure. The solid residue was re-dissolved in 50 ml of a H$_2$O - MeCN - n-BuOH 1 : 1 : 2 mixture and 5 g of silanized Silica-gel 60 (0.06 ~ 0.2 mm; Merck) was added under stirring. After 30 minutes, the solvents were evaporated at 45°C under reduced pressure and the solid residue was loaded on a column of 35 g of the same above silanized Silica-gel in H$_2$O. The column was developed with a linear gradient from 10 to 70% of MeCN in H$_2$O in 15 hours at the flow-rate of 100 ml/hour, while collecting 10 ml-fractions which were checked by HPLC. Those fractions containing pure title compound were pooled and n-BuOH (v/v) was added. The resulting solution was concentrated, at 40°C under reduced pressure, to a small volume (about 10 ml), and then Et$_2$O (100 ml) was added. The precipitated solid was collected and slurried with 20 ml of DMSO. The resulting suspension was filtered and the clear filtrate was lyophilized, yielding 0.11 g of pure title compound (HPLC: Method A, Rt 22.6 minutes).

$C_{60}H_{50}N_6O_{16}Cl_2$, MW 1182.0; ESI-MS: m/z 1181.3, [M + H]$^+$

Phe^3-TDHP, Methyl Ester (5a)

A solution of 1.8 g (about 1.5 mmol) of the above compound (4a) in 120 ml of a MeOH - 1 N HCl - DMF 6 : 2 : 1 mixture was hydrogenated (1 atm, 25°C) in the presence of 1.5 g of 5% Pd/C. The catalyst was filtered off and MeOH was evaporated at 35°C under reduced pressure, then 100 ml of H$_2$O was added and the resulting solution extracted with 150 ml of EtOAc. The organic layer was discarded and the resulting aqueous suspension was extracted with 130 ml of n-BuOH. The butanolic phase was separated and concentrated at 30°C under reduced pressure to a volume of about 20 ml. On adding 150 ml of Et$_2$O the precipitated solid was collected (0.65 g, HPLC: Method A, Rt 17.5 minutes, titre about 75%) and used for the next step without any further purifica-

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[††††] Average mass.
[†††††] Monoisotopic mass.

tion.

Di-BOC-D-Lys^1-Phe^3-TD-Heptapeptide, Methyl Ester (6a)

To a stirred solution of 0.29 g of crude product 5a in 3 ml of DMF, 0.07 ml of TEA and 0.19 g of di-BOC-D-Lysine, N-hydroxy-succinimide ester were added at room temperature. After 1 day reaction, 50 ml of H$_2$O was added and the resulting suspension was adjusted at pH 3 with 1 N HCl, then it was extracted with 50 ml of n-BuOH. The organic layer was separated, washed with 25 ml of H$_2$O, then it was concentrated at 45°C under reduced pressure to a small voume (about 3 ml). On adding Et$_2$O (30 ml), the precipitated solid was collected (0.27 g, HPLC: Method A, Rt 22.5 minutes, titre about 45%) and used without any purification for the final step.

(D-Lys)^1-(Phe)^3-TD-DHP (DalbaHePtide), Methyl Ester (7a), MDL 63,166

The above crude product (0.27 g) was dissolved at 10°C in 5 ml of dry TFA. After 10 minutes, the solvent was evaporated at 15°C under reduced pressure. The oily residue was re-dissolved in 30 ml of a H$_2$O - MeOH 1 : 1 mixture and the resulting solution was adjusted at pH 3.5 with 1 N NaOH, then it was loaded on a column of 50 g of silanized Silica-gel. Column chromatography was performed as previously described obtaining 0.05 g of pure title compound (HPLC: Method A, Rt 18.3 minutes, titre 97%).

Preparation of MDL 64,945

N^2-CBZ-(N$_\alpha$-FMOC-N$_\varepsilon$-BOC-L-Lysyl)^3-TDPP, Methyl Ester (2b)

To a stirred solution of 500 mg (0.43 mmol) of compound 1 (TFA salt) in 10 ml of DMF, 0.055 ml (0.5 mmol) of N-methylmorpholine and a solution of 355 mg (0.56 mmol) of N$_\alpha$-FMOC-N$_\varepsilon$-BOC-L-lysine pentafluorophenyl ester in 5 ml of DMF were added at 5°C. After stirring at 5°C for 3 hours and at room temperature for 13 hours, 100 ml of H$_2$O was added and the resulting suspension was adjusted at pH 3 with 1 N HCl. After extraction with n-BuOH (30 ml × 2), the organic extract was washed sequentially with 15 ml of H$_2$O, saturated NaHCO$_3$ solution (15 ml × 2), H$_2$O adjusted at pH 3 (10 ml × 2), and H$_2$O (10 ml × 2), and then it was evaporated to yield 610 mg (0.41 mmol) of the title compound (HPLC: C system, Rt 18.1 minutes).

C$_{77}$H$_{73}$N$_7$O$_{21}$Cl$_2$, MW 1503.4; ESI-MS: m/z 1502.4, [M + H]$^+$

N^2-CBZ-(N$_\varepsilon$-BOC-Lys)^3-TDPP, Methyl Ester (3b)

A solution of 600 mg (0.40 mmol) of 2b in 5 ml of piperidine was stirred at room temperature for 2 hours, afterwards piperidine was evaporated at 40°C under reduced pressure. The oily residue was stirred with Et$_2$O to give a solid compound, which was collected and

re-dissolved in a n-BuOH - H$_2$O 1 : 1 mixture (100 ml). The organic layer was sequentially washed with H$_2$O adjusted at pH 3 (20 ml × 3), H$_2$O (10 ml), a 2% solution of N-methylmorpholine in H$_2$O (10 ml) and H$_2$O until the pH of the aqueous layer was 7.5. The butanolic solution was evaporated at 40°C under reduced pressure to yield 480 mg (0.375 mmol) of the title compound (HPLC: C system, Rt 5.4 minutes).

C$_{62}$H$_{63}$N$_7$O$_{19}$Cl$_2$, MW 1281.1; ESI-MS: m/z 1280.4, [M + H]$^+$

N^2-CBZ-(N$_\varepsilon$-BOC-Lys)^3-TDHP, Methyl Ester (4b)

To a stirred solution of 240 mg (0.19 mmol) of compound 3b in 250 ml of a DMF - CH$_2$Cl$_2$ 1 : 4 mixture was added 140 mg (0.5 mmol) of HOBT hydrate followed by 16 mg (0.08 mmol) of DCC. The reaction mixture was vigorously stirred at room temperature overnight, and then a new portion of 16 mg (0.08 mmol) of DCC was added. Stirring was continued for 36 hours and then a new portion of 16 mg (0.08 mmol) of DCC was added. The reaction mixture was stirred for additional 36 hours and then the solvents were evaporated at 30°C under reduced pressure to give an oily residue, which was suspended in a H$_2$O - MeCN 1 : 1 mixture (10 ml) and left at 5°C for 3 hours. Insoluble dicyclohexylurea was filtered off and the clear filtrate was evaporated to a half of the initial volume. After adding 50 ml of n-BuOH, the organic layer was sequentially washed with H$_2$O adjusted at pH 3 with 1 N HCl (15 ml × 2), H$_2$O (15 ml × 2), saturated NaHCO$_3$ solution (15 ml × 2) and H$_2$O (10 ml × 3). Afterwards, the butanolic solution was concentrated at 40°C under reduced pressure to a small volume (about 5 ml). On adding Et$_2$O (50 ml) the precipitated solid was collected to yield 180 mg of crude 4b which was re-dissolved in MeOH (3 ml) and loaded on a column (3 × 70 cm) of LH-20 Sephadex pre-equilibrated with MeOH. The column was slowly developed with MeOH at a rate of 2 ml/hour, while collecting 1 ml-fractions. Those fractions containing 4b (HPLC) were pooled and the solvent was evaporated at 35°C under reduced pressure. Purification on LH-20 column was repeated to give 75 mg (0.06 mmol) (31%) of pure 4b (HPLC: C system, Rt 11.0 minutes).

C$_{62}$H$_{61}$N$_7$O$_{18}$Cl$_2$, MW 1263.1; ESI-MS: m/z 1262.4, [M + H]$^+$

(N$_\varepsilon$-BOC-Lys)^3-TDHP, Methyl Ester (5b)

A solution of 4b (75 mg, 0.06 mmol) in a MeOH - AcOH 20 : 1 mixture (7 ml) was hydrogenated over 5% Pd/C at 1 atm and 25°C. The catalyst was filtered off, the solvents were evaporated at 35 °C under reduced pressure to a volume of ∼2 ml to which 20 ml of Et$_2$O was added to yield 55 mg (0.05 mmol) of 5b (HPLC: C system, Rt 10.3 minutes).

C$_{54}$H$_{55}$N$_7$O$_{16}$Cl$_2$, MW 1129.0; ESI-MS: m/z 1128.3, [M + H]$^+$

(Di-BOC-D-Lys)^1-(N_ε-BOC-Lys)^3-TD-Heptapeptide, Methyl Ester (6b)

To a stirred solution of 55 mg (0.05 mmol) of 5b in 2 ml of DMF, 0.02 ml (0.15 mmol) of TEA and 55 mg (0.125 mmol) of di-BOC-D-lysine, N-hydroxysuccinimide ester were added at room temperature. After 18 hours stirring, 20 ml of H_2O was added and the resulting suspension was adjusted at pH 3 with 1 N HCl. The suspension was extracted with EtOAc (10 ml × 2) and the organic layer was washed with water (10 ml × 2), afterwards it was concentrated at 45°C to a small volume (about 1.5 ml). On adding Et_2O (30 ml), the precipitated solid was collected (50 mg; HPLC: C system, Rt 13.9 minutes) and used without purification for the preparation of 7b.

(D-Lys)^1-(Lys)^3-TD-DHP, Methyl Ester (7b), MDL 64,945

Crude product 6b (50 mg) was dissolved at 10°C in 1.5 ml of dry TFA. After 10 minutes, Et_2O (30 ml) was added and the precipitated solid (50 mg) was collected; 7b was purified by preparative HPLC using a linear gradient MeCN - 0.01 M H_3PO_4, pH 2.6 (2→40% of MeCN, 35 minutes, flow rate 4.0 ml) to give 14 mg of pure 7b (HPLC: B system, Rt 8.3 minutes, titre 95%).

(N_α-BOC-N-Me-D-Leu)^1-(N_ε-BOC-Lys)^3-TD-Heptapeptide, Methyl Ester (6c)

To a stirred solution of 55 mg (0.05 mmol) of 5b in 2 ml of DMF, a solution of 40 mg (0.16 mmol) of N-Me-N-BOC-D-Leu-HOBT (prepared in situ) in 0.6 ml of DMF was added at room temperature. The reaction mixture was stirred at room temperature overnight, afterwards 8 ml of a n-BuOH - H_2O 1 : 1 mixture was added. The organic layer was washed sequentially with H_2O adjusted to pH 3 with aq. HCl, H_2O, 5% NaHCO$_3$ solution and H_2O until pH 7 was reached. The butanolic phase was evaporated at 35°C under reduced pressure to a volume of about 0.2 ml. Addition of 30 ml of Et_2O gave a precipitated solid (52 mg), which was collected and purified by column chromatography (10 g of LH-20) in MeOH, the procedure being repeated twice. The substance obtained was purified by HPLC (linear gradient MeCN - 0.2% ammonium formate, pH 3.8: 20→80% of acetonitrile, 40 minutes, flow-rate 1.0 ml) obtaining 8.5 mg (0.005 mmol) of pure 6c (HPLC, D system, Rt 30.6 minutes, titre 89%).

$C_{66}H_{76}N_8O_{19}Cl_2$, MW 1356.3; ESI-MS: m/z 1355.5, [M + H]$^+$

(N-Me-D-Leu)^1-(Lys)^3-TD-DHP, Methyl Ester (7c), MDL 64,468

Compound 6c (8 mg, 0.005 mmol) was dissolved at room temperature in 0.3 ml of dry TFA. After 15 minutes, the solvent was evaporated at 20°C under reduced pressure to about 0.1 ml volume. Addition of Et_2O (2 ml) resulted in a precipitated solid, which was collected to yield 6 mg of 7c (HPLC, D system, Rt 8.6 minutes, 95%

titre).

N^2-CBZ-(N_α-BOC-N_{im}-BOM-Histidyl)^3-TDPP, Methyl Ester (2d)

Compound 2d (100 mg; HPLC: D system, pH 6.4; Rt 9.12 minutes) was prepared in 65% yield starting from 1 (125 mg, 0.11 mmol) and N_α-BOC-N_{im}-BOM-histidine pentafluorophenyl ester (112 mg, 0.22 mmol) by the method previously described for the preparation of 2b.

N^2-CBZ-(N_{im}-BOM-His)^3-TDPP, Methyl Ester (3d)

Compound 3d (HPLC: C system, Rt 5.03 minutes) was prepared following a same procedure as that used for compound 3b, starting from 2d, in 84% yield.

$C_{65}H_{58}N_8O_{18}Cl_2$, MW 1310.1; ESI-MS: m/z 1309.3, [M + H]$^+$

N^2-CBZ-(N_{im}-BOM-His)^3-TDHP, Methyl Ester (4d)

Compound 4d was prepared from 3d (84 mg) following method described for the preparation of 4b. After purification on LH-20 column in MeOH, 11 mg of crude title compound was obtained, which gave after preparative HPLC (linear gradient MeCN - 0.2% ammonium formate, pH 6.4: 10→75% of MeCN, 50 minutes, flow-rate 1.0 ml) 2.5 mg (0.002 mmol) of 4d (HPLC: C system, Rt 12.65 minutes).

$C_{65}H_{56}N_8O_{17}Cl_2$, MW 1292.1; ESI-MS: m/z 1291.3, [M + H]$^+$

N-Methyl-N-BOC-D-Leucine, N-hydroxybenzotriazole Ester

To a stirred solution of 41 mg (0.165 mmol) of N-methyl-N-Boc-D-leucine in 0.6 ml of dry DMF, 47 mg (0.33 mmol) of HOBT hydrate was added at 5 °C followed by 34 mg (0.165 mmol) of DCC. The reaction mixture was kept at 5°C for 16 hours and then it was filtered to remove a white precipitate, which was discarded. A pure solution of the title compound was used in the synthesis of 5b.

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