Solid-Phase Synthesis of the Cyclic Liponanadepsipeptide [*N*-Mst(Ser1), D-Ser4, L-Thr6, L-Asp8, L-Thr9]Syringotoxin**

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Abstract: An optimized solid-phase strategy for the preparation of the cyclic liponanadepsipeptide [*N*-Mst(L-Ser1), D-Ser4, L-Thr6, L-Asp8, L-Thr9]syringotoxin is reported. The strategy is based on the use of a mild orthogonal protection scheme and the incorporation of the nonproteinogenic amino acid (*Z*)-Dhb into the peptide chain as the dipeptide Fmoc-Thr(*t*Bu)-(*Z*)-Dhb-OH. The didehydrodipeptide was synthesized by a water-soluble carbodiimide-induced β-elimination of a protected dipeptide containing a residue of Thr with its free hydroxy side chain unprotected.

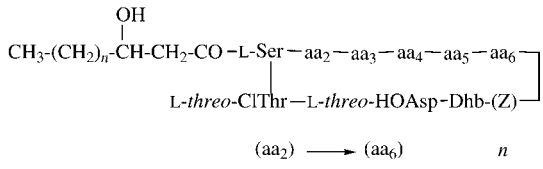
Keywords: antimicrobial peptides · cyclic peptides · didehydroamino acids · *Leishmania* · peptides · protecting groups

Introduction

Depsipeptides incorporate a wide variety of structures within their sequence and these include hydroxyamino acids involved in lactone bonds. Extensive amino acid modifications, including derivatization with fatty acids and formation of cyclic structures, are also very frequent features of this type of system. The main natural sources of these materials are bacteria, fungi, and marine invertebrates. These compounds act as virulence factors in phytopathology[1] or as components of defensive mechanisms for the organisms that produce them. More importantly, depsipeptides show great potential as antitumor, antifungal, or antiviral agents.[2, 3]

These depsipeptides incorporate extensive amino acid modifications and as a consequence their recombinant production is usually quite difficult. For this reason, chemical synthesis constitutes an important alternative approach to the discovery of structure–activity relationships for these peptides despite the lack of many commercial amino acid analogues.

The cyclic liponanadepsipeptide syringotoxin is a component of the phytotoxin family (syringomycin, syringostatin, syringotoxin, pseudomycin) produced by the plant pathogenic bacteria *Pseudomonas syringae syringae* (Figure 1)[1]. All



	(aa$_2$) → (aa$_6$)	*n*
syringomycin	D-Ser-D-Dab-L-Dab-L-Arg-L-Phe	10
syringostatin	D-Dab-L-Dab-D-Hse-L-Orn-L-*a*-Thr	8
syringotoxin	D-Dab-Gly-D-Hse-L-Orn-L-*a*-Thr	10
pseudomycin	D-Dab-L-Asp-L-Lys-L-Dab-L-*a*-Thr	2

Figure 1. Cyclic liponanadepsipeptides isolated from *Pseudomonas syringae syringae*.

members of this family share common characteristics such as a cluster of three uncommon amino acids ((*Z*)-2,3-didehydro-2-aminobutyric acid ((*Z*)-Dhb), 4-L-*threo*-chlorothreonine, and 3-L-*threo*-hydroxyaspartic acid) at the *C*-terminal part[4] of the nonapeptide moiety and, in addition, the terminal carboxyl group forms a large lactone ring with the hydroxy group of the *N*-terminal serine, which in turn is *N*-acylated by a long chain 3-β-hydroxy fatty acid.

Syringotoxin is a membrane-active antifungal depsipeptide.[5] Our group has assayed the lethal activity of this

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[**] Abbreviations used for amino acids and the designations of peptides follow the rules of the IUPAC–IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* **1972**, *247*, 977–983. Amino acid symbols denote the L-configuration unless stated otherwise. All solvent ratios are volume/volume (*v*/*v*) unless stated otherwise.

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compound on the human protozoal parasite *Leishmania*,[6] a scourge in tropical and subtropical countries where the development of new agents is urgently needed due to the growing incidence of resistance against classical antibiotics and the frequent and severe side effects associated with the drugs used today.[7]

As a first step towards the development of an efficient solid-phase synthetic approach to syringotoxin and other members of this family, we synthesized a simplified analogue of syringotoxin in which the main structural features of the natural peptide were retained, for example, *N*-terminal derivatization with a long-chain fatty acid, formation of the cyclic nonapeptide through a lactone bond, and the stereochemistry of the C_α atoms of the peptide.

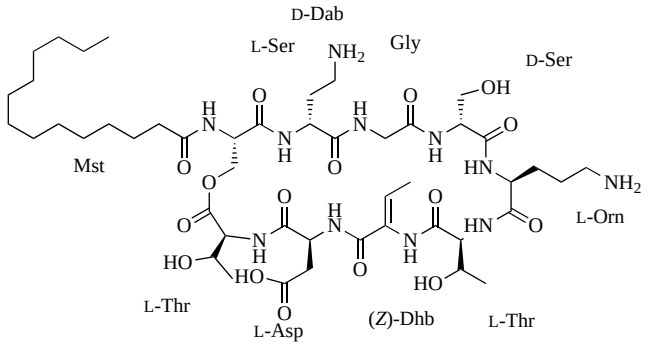
The differences between the synthetic analogue and the natural syringotoxin were as follows: 1) the two uncommon residues 4-L-*threo*-chlorothreonine and 3-L-*threo*-hydroxyaspartic acid were replaced by their proteinogenic analogues Thr and Asp, respectively; 2) the 3-hydroxytetradecanoic acid moiety was replaced by a nonsubstituted myristic acid (Mst); and 3) D-Ser and Thr replaced D-homoserine (D-Hse) and L-*allo*Thr, respectively. Thus, the new analogue was accordingly named [*N*-Mst(Ser1), D-Ser4, L-Thr6, L-Asp8, L-Thr9]syringotoxin (Figure 2).

Results and Discussion

Our synthetic strategy for [*N*-Mst(Ser1), D-Ser4, L-Thr6, L-Asp8, L-Thr9]syringotoxin is based on a solid-phase approach to the elongation of the peptide chain,[8] cleavage of the protected peptide from the resin, subsequent cyclization, and final deprotection (Scheme 1).

Analysis of the synthetic route shows the cornerstones of this approach to be: 1) Use of a fluorenylmethoxycarbonyl (Fmoc)-based strategy due to the high acid lability of residues in syringotoxin, which prevents the use of strong acids, such as HF or trifluoromethanesulfonic acid, to remove the protecting groups. 2) Use of the Barlos (2-chlorotrityl chloride) resin,[9] which minimizes the formation of diketopiperazines and allows the cleavage of the peptide under very mild conditions and in the presence of other acid-labile protecting groups. 3) Cyclization through an amide bond between the Gly carboxy function and the amino group of the D-Ser. The choice of this cyclization point should be advantageous because it allows the suppression of epimerization (Gly as *C*-carboxyl component) and reduces the steric hindrance, which should increase the cyclization yield. Alternative cyclization routes involving the presence of much more hindered amino acids or an ester linkage were rejected due to the lower reactivity of the hydroxy group relative to the amino group. Furthermore, cyclization through the ester would preclude the presence of other free hydroxy groups in the molecule, as planned in the synthesis of syringotoxin, in which 3-hydroxyaspartic and 3-hydroxytetradecanoic acids would be incorporated with unprotected hydroxyl groups due to the difficult nature of their syntheses. 4) Addition of the nonproteinogenic residue (*Z*)-Dhb into the peptide chain as the dipeptide Fmoc-Thr(*t*Bu)-(*Z*)-Dhb-OH. The α-amino group of residue (*Z*)-Dhb is inefficient at forming an amide linkage because it participates in a tautomeric amino–imino equilibrium. However, the solution-phase synthesis of the dipeptide Fmoc-Thr(*t*Bu)-Thr-OH and subsequent dehydration of the *C*-terminal Thr residue avoids this problem. 5) The serine residue plays a key role in the sequence because all its functional groups take part in bonding in the peptide chain. Thus, besides forming the peptide bond with D-α,γ-diaminobutyric acid (D-Dab), its α-amino group allows addition of myristic acid and its hydroxy group forms the ester linkage with the carboxylic acid function of the Thr residue. The initial formation of the ester bond is preferred because it avoids the necessity of protection of the hydroxy group of the fatty acid in the synthesis of syringotoxin. The use of this strategy will require the introduction of the Ser residue with the α-amino function protected with the allyloxycarbonyl (Alloc) group, which is orthogonal to the Fmoc and acid-labile protecting groups and will therefore preserve the Fmoc group for the protection of the Thr residue.

To carry out the process outlined in Scheme 1, Alloc-Ser-OH and the didehydropeptide Fmoc-Thr(*t*Bu)-(*Z*)-Dhb-OH first had to be synthesized. Alloc-Ser-OH was prepared by using the trimethylsilyl (TMS) group as a temporary protect-



syringotoxin analogue	Mst	L-Ser	D-Dab	Gly	D-Ser	L-Orn	L-Thr	(*Z*)-Dhb	L-Asp	L-Thr
syringotoxin	β-HO-tetradecanoic acid	L-Ser	D-Dab	Gly	D-Hse	L-Orn	L-*a*Thr	(*Z*)-Dhb	L-*threo*-HOAsp	L-*threo*-ClThr

Figure 2. [*N*-Mst(Ser1), D-Ser4, L-Thr6, L-Asp8, L-Thr9]syringotoxin and comparison with the natural sequence.

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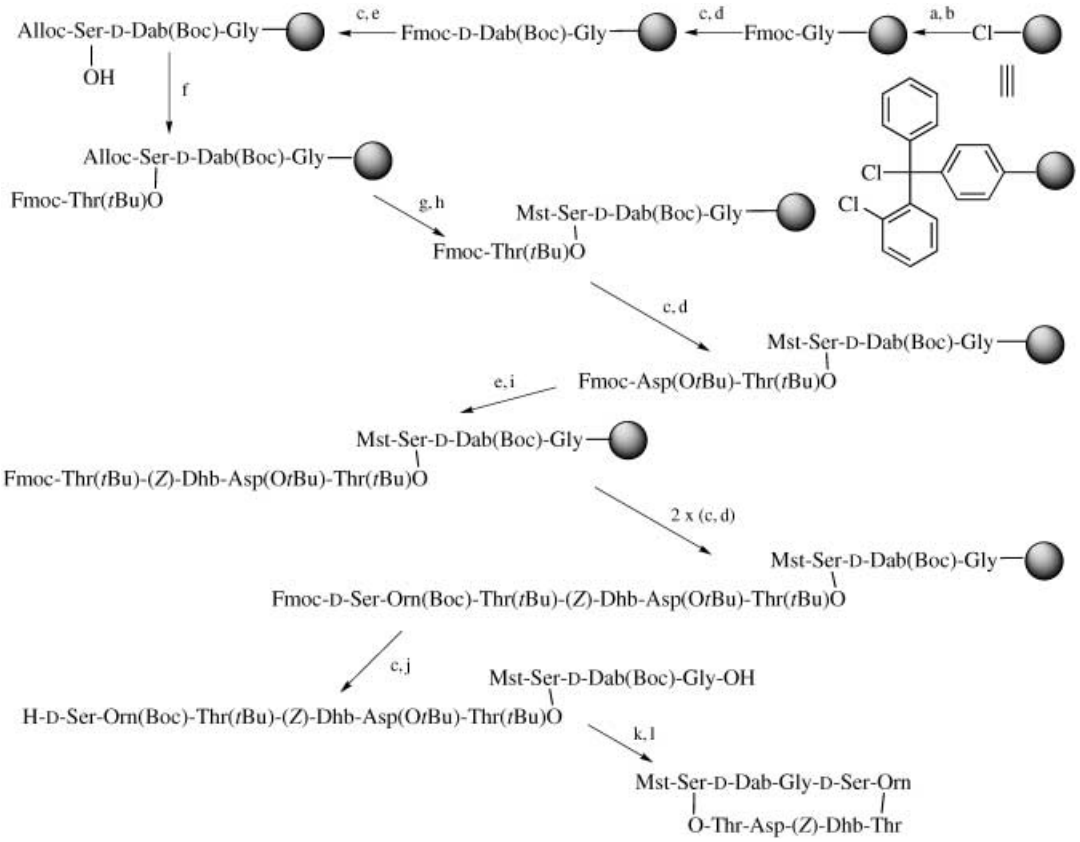

Scheme 1. Solid-phase strategy developed for the preparation of [*N*-Mst(Ser1), D-Ser4, L-Thr6, L-Asp8, L-Thr9]syringotoxin. a) Fmoc-Gly-OH, DIEA, CH_2Cl_2; b) MeOH; c) piperidine/DMF (2:8); d) Fmoc-aa-OH, DIPCDI/HOBt, DMF; e) Alloc-Ser-OH, TBTU/HOBt/DIEA, DMF; f) Fmoc-Thr(*t*Bu)-OH, DIPCDI-DMAP, DMF; g) $[Pd(PPh_3)_4]$, $PhSiH_3$, CH_2Cl_2; h) Mst-OH, DIPCDI/HOBt, DMF; i) Fmoc-Thr(*t*Bu)-(*Z*)-Dhb-OH, DIPCDI/HOAt, DMF; j) TFA/ CH_2Cl_2 (1:99); k) DIPCDI/HOBt/DIEA (4:4:3), CH_2Cl_2; l) TFA/H_2O (19:1).

ing group for all functional positions to avoid the undesired formation of side products such as di- and tripeptides.[10, 11] Thus, Ser was treated with trimethylsilyl chloride in CH_2Cl_2 in the presence of *N*,*N*-diisopropylethylamine (DIEA), and the intermediate product was treated in situ with allyl chloroformate. Finally, removal of the remaining protecting TMS groups occurred on aqueous workup to yield the final product.

As explained above, didehydropeptide Fmoc-Thr(*t*Bu)-(*Z*)-Dhb-OH represents the best way to insert the non-proteinogenic residue (*Z*)-Dhb into a peptide chain. Unfortunately, in this case the formation of the double bond in the didehydroamino acid on the solid-phase cannot be performed through a β-elimination reaction from the Thr residue with a free hydroxy function—a process developed previously in our laboratory.[12] This method cannot be used because syringotoxin contains other residues that can also undergo dehydration (e.g., ClThr, HOAsp, and 3-OH-Mst). We have previously prepared didehydrodipeptides in solution with either Boc/allyl or Alloc/*t*Bu protecting groups for the α-amino function (semipermanent protection) and *C*-carboxyl function (temporary protection).[13] Although Fmoc is perfectly stable to dehydration conditions,[13] the use of the Fmoc/*t*Bu derivative did not lead to the Fmoc-didehydrodipeptide for reasons that are unclear. In the present work, *tert*-butyloxycarbonyl (Boc) protection is not compatible with either of the other side chain protecting groups or the Barlos resin. Furthermore, *t*Bu protection is not compatible with the presence of the *N*-terminal Thr in the dipeptide, which also requires *t*Bu protection. Thus, we were forced to attempt the preparation of Fmoc-Thr(*t*Bu)-(*Z*)-Dhb-OH by dehydration of Fmoc-Thr(*t*Bu)-Thr-Oallyl, which is best prepared from Fmoc-Thr(*t*Bu)-OSu and HCl · H-Thr-Oallyl in the presence of DIEA.[14, 15] The dehydration was successfully accomplished by using a modification of the method developed in our laboratory for the preparation of (*Z*)-Dhb-containing peptides on solid-phase,[11] which was based on an initial report by Fukase et al.[16] This method uses 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (EDC; 2 equiv) as an activating reagent for the hydroxyl function in the presence of CuCl (1.2 equiv) in CH_2Cl_2/ *N*,*N*-dimethylformamide (DMF) (98:2) under a nitrogen atmosphere for 20 h.[17] Finally, removal of the allyl group was performed with $[Pd(PPh_3)_4]$ (0.03 equiv) in the presence of $PhSiH_3$ (3 equiv) under argon for 2 h.[18] The resulting target dipeptide was ready to be incorporated into the peptide sequence. This strategy allowed the preparation of the *Z* isomer with excellent stereoselectivity (97:3 *E*:*Z*).[19]

A limited incorporation of Fmoc-Gly-OH (0.7 mmol per g resin) onto Barlos resin was performed in the presence of DIEA (10.5 mmol per g resin).[20, 21] Peptide chain elongation was carried out by using an Fmoc/*t*Bu strategy. Removal of the Fmoc group was achieved with piperidine/DMF (2:8). The majority of subsequent couplings were performed with *N*,*N*′-diisopropylcarbodiimide (DIPCDI)/ 1-hydroxybenzotriazole

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(HOBt) as the coupling reagent; however, the incorporation of the third amino acid (Alloc-Ser-OH), Fmoc-Thr(*t*Bu)-OH, and the dipeptide Fmoc-Thr(*t*Bu)-(*Z*)-Dhb-OH was carried out by following other strategies. Thus, Alloc-Ser-OH was incorporated by using *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium tetrafluoroborate *N*-oxide (TBTU)/DIEA, which is a very powerful coupling reagent. This approach was used to decrease the reaction time and, therefore, the time during which the resin-anchored *N*-terminal unprotected dipeptide existed, with the aim of minimizing the formation of diketopiperazine (DKP).[22] The addition of Fmoc-Thr(*t*Bu)-OH to form the ester linkage was carried out using DIPCDI and a catalytic amount of 4-dimethylaminopyridine (DMAP).[23] This procedure gave a quantitative yield according to the alcohol test.[24] The carboxyl function of Fmoc-Thr(*t*Bu)-(*Z*)-Dhb-OH is less reactive than the corresponding group in carbamate-protected amino acids. Therefore, 1-hidroxy-7-azabenzotriazole (HOAt)[25] was used instead of HOBt.[26] The Alloc group was removed by using $[Pd(PPh_3)_4]$ (0.1 equiv) in the presence of $PhSiH_3$ (10 equiv) as a scavenger under argon.[17, 27]

The cleavage of the protected peptide from the resin was carried out smoothly with trifluoroacetic acid (TFA)/CH_2Cl_2 (1:99) (5×30 s) and the crude product was evaluated by reversed-phase HPLC. The cyclization step was carried out at a concentration of 1 mM in CH_2Cl_2 with DIPCDI/HOBt/DIEA.[28] DIEA was added to neutralize the trifluoroacetate salt and avoid undesired trifluoroacetylation.[29] In a first approach, the protected cyclic peptide was treated with TFA/H_2O (95:5) with the aim of purifying the final peptide. However, the unprotected final cyclic peptide is extremely insoluble and this precludes its purification in this way. Finally, the protected cyclic peptide was purified by MPLC (Figure 3a), followed by the removal of the *t*Bu-based protecting groups with TFA/H_2O (95:5) (Figure 3b). The final [*N*-Mst(Ser1), D-Ser4, L-Thr6, L-Asp8, L-Thr9]syringotoxin was then obtained by washing the crude product with Et_2O (Figure 3c).

This peptide was tested on *Leishmania donovani* promastigotes and the resulting synthetic lipodepsipeptide only showed a mild leishmanicidal activity; on the other hand, the LD_{50} value (LD_{50} lethal dose 50 (concentration at which parasite poliferation was reduced by 50%)) and its 95% confidence interval, given in parentheses, for the natural product was 3.9(14.8–3.1) μM, which increased to 19.4(51.2–0.13) μM in the synthetic analogue. A study of the samples by optical microscopy showed that at concentrations over 25 μM, parasites first appeared swollen and after 1 h extensive lysis was observed, pointing towards a colloid–osmotic shock effect as the main mechanism for the leishmanicidal activity—a characteristic also described for natural syringotoxin.[5]

Accordingly, the reported leishmanicidal activities for the natural and synthetic analogues differed widely. Two non-mutually exclusive aspects may account for this result: firstly, the harsh conditions required for the synthesis and cleavage of the synthetic product from the support may lead to the formation of aggregates, a possibility that is consistent with the poor solubility of the synthetic analogue in aqueous media. Secondly, this process may lead to the formation of aggregates with a stoichiometry that makes it difficult for them to partition into the parasite membrane. In both cases the outcome will be the same, that is, a marked reduction in the real active concentration of the product.

An alternative approach deals with modifications introduced into the natural structure of syringotoxin. The inclusion of natural L-threonine instead of the 4-chlorinated analogue may be at least partially responsible for the loss of activity. A general explanation for the role of halide atoms within peptide molecules is still unclear. Halide atoms will increase the hydrophobicity of the structure and, consequently, their ability to partition into biological membranes. On the other hand, as bulky atoms they will impose severe steric hindrance that could affect the population of molecules in an active conformation. The presence of halide atoms within the structure of antibiotic peptides is quite rare. For example, in terms of eukaryotic antibiotic peptides, 4-chlorothreonine was described in the depsipeptide actinomycin Z,[30] and a 6-chloro-*N*-methyltryptophan was reported in the cyclic peptide keramamide L, which was isolated from a sponge and showed cytotoxicity against tumor cells.[31] Nevertheless, an analysis of the effect of the presence of halides on antibiotic activity has not been reported. A 6-brominated tryptophan was reported in a linear antibiotic peptide from hagfish[32] and in styelin D, which is another linear peptide purified from the solitary tunicate Ascidia.[33] In the latter peptide, the presence of the brominated tryptophan increases the biological activity over a wider range of salinity or pH values.[33] When *P. syringae syringae* was grown in the absence of halide ions, a deschlorosyringotoxin was obtained. This analogue suffered from a three-fold decrease in antifungal activity relative to the parent compound.[34]

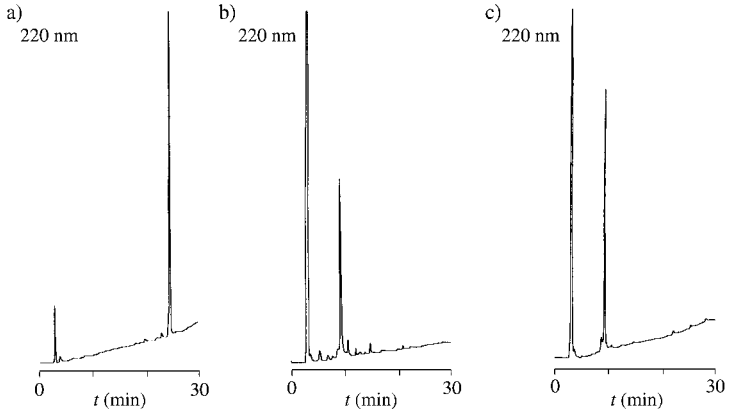

Figure 3. HPLC chromatograms of a) purified cyclic protected peptide; b) crude of final deprotected peptide; c) final product. Reverse-phase C_4 columns were used for the analysis with elution by a linear gradient over 30 min of 0.036% TFA in CH_3CN and 0.045% TFA in H_2O from 35:65 to 100:0, flow rate 1.0 mL min^{-1}.

The substitution of the 3-hydroxymyristic acid by the parent structure would not greatly affect the overall hydrophobicity or amphipathicity of the molecule. Although fatty acid acylation of antibiotic peptides frequently leads to an increase in their antibiotic activity,[35, 36] this outcome depends mainly on the position and length of the fatty acid chain, as reported for a set of acylated CA(1–7)M(2–9) analogues. The differences in the leishmanicidal activities of these compounds became less marked for fatty acid lengths over 12 carbon atoms.[35] Nevertheless, the 3-hydroxy structure is a consistent feature of all the members of this phytotoxin family, whereas the length of the fatty acid chain is not.[1] It is therefore possible that this factor could play a more specific role, which is not apparent in this analogue, than the slight modification of the overall physical parameters of the molecule.

Finally, one would not expect substitution of D-Hse and L-*allo*Thr by D-Ser and Thr to be particularly significant if a membrane perturbation effect were merely responsible for leishmanicidal activity. However, for the closely related syringomycin, fungicidal activity requires the presence of ergosterol[37] and sphingolipids with α-hydroxylated very long chain fatty acids[38] on the membrane of the target organism. At present it is unclear whether this lipodepsipeptide acts by modulation of the physical properties of the membrane or by specific interactions with particular membrane components. If the latter case is true, perhaps the analogue is devoid of the recognition features required for this interaction. Furthermore, although the cyclic structure and *N*-terminal fatty acid derivatization preclude degradation by exopeptidases,[39] changes into natural amino acids may sensitize the depsipeptide structure towards endopeptidase degradation; indeed, one of the most abundant proteins in the plasma membrane of Leishmania promastigotes is leishmaniolysin,[40] an endopeptidase with a broad substrate specificity that could degrade syringotoxin activity.

Conclusion

In conclusion, an optimized solid-phase strategy for the preparation of an analogue of the antimicrobial peptide syringotoxin is reported. This approach is based on the use of an orthogonal protection scheme, as well as the incorporation of the nonproteinogenic amino acid (*Z*)-Dhb into the peptide chain as the dipeptide Fmoc-Thr(*t*Bu)-(*Z*)-Dhb-OH. The synthesis of peptides with structures similar to the natural peptide will be needed to obtain syringotoxin analogues with therapeutic applications. However, this work clearly demonstrates the feasibility of synthesizing this class of phytotoxin and the method could be easily extended to include other members of the lipodepsipeptides.

Experimental Section

General methods: Protected amino acid derivatives, HOBt, and TBTU were obtained from Applied Biosystems (Framingham, MA), Bachem (Bubendorf, Switzerland), Albatross (Montreal, Canada), and NovaBiochem (Läufelfingen, Switzerland). Barlos resin was obtained from CBL–Patras (Patras, Greece). DIEA, DIPCDI, piperidine, TFA, and DMAP were obtained from Aldrich (Milwaukee, WI), and EDC·HCl was a gift from Luxembourg Industries (Tel Aviv, Israel). DMF, CH_2Cl_2, and EtOAc were obtained from SDS (Peypin, France). Acetonitrile (HPLC grade) was supplied by Scharlau (Barcelona, Spain), and hexane, Et_2O, and methanol were obtained from Panreac (Barcelona, Spain). All commercial reagents and solvents were used as received with the exception of DMF and CH_2Cl_2, which were bubbled with nitrogen to remove volatile contaminants (DMF) and stored over activated 4 Å molecular sieves (Merck, Darmstadt, Germany) (DMF) or $CaCl_2$ (CH_2Cl_2). Et_2O was stored over Na.

Solution reactions were performed in round-bottomed flasks. Organic solvent extracts were dried over anhydrous $MgSO_4$, followed by solvent removal under reduced pressure at temperatures below 40 °C.

Solid-phase syntheses were performed in polypropylene syringes (10 mL) fitted with a polyethylene porous disc. Solvents and soluble reagents were removed by suction. Removal of the Fmoc group was carried out with piperidine/DMF (2:8, *v*/*v*) (1 × 1 min, 3 × 5 min, 1 × 10 min). Washings between deprotection, coupling, and final deprotection steps were carried out with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min) using 10 mL solvent g^{-1} resin for each wash. Peptide synthesis transformations and washes were performed at 25 °C.

HPLC columns (Nucleosil C_{18} and C_4 reversed-phase column, 250 × 40 mm, 10 μm) were obtained from Scharlau (Barcelona, Spain). Analytical HPLC was carried out on a Shimadzu instrument comprising two solvent delivery pumps (model LC-6A), automatic injector (model SIL-6B), variable wavelength detector (model SPD-6A), system controller (model SCL-6B), and plotter (model C-R6A). UV detection was performed at 220 nm and linear gradients of CH_3CN (+0.036% TFA) into H_2O (+0.045% TFA) were run at 1.0 mL min^{-1} flow rate from: (condition A, C_{18}) 0:1 to 1:0 over 30 min; (condition B, C_{18}) 3:7 to 10:0 over 30 min; (condition C, C_{18}) 2:8 to 10:0 over 30 min; (condition D, C_4) 3:7 to 10:0 over 30 min; (condition E, C_4) 35:65 to 1:0 over 30 min. Flash chromatography was carried out using silica gel 60 (35–70 μm) SDS (Peypin, France).

IR spectra were obtained by using a Nicolet 510 FT-IR spectrophotometer. MALDI-TOF and ES(+)-MS analyses of peptide samples were performed on an Applied Biosystems Voyager DE RP, using 2,5-dihidroxybenzoic acid (DHB) matrix, and in a Micromass VG-quattro spectrometer. CI-MS analyses of amino acid derivatives were performed with a Hewlett–Packard HP-5988A spectrometer. ^{1}H NMR (600, 400, 300, 200 MHz) and ^{13}C NMR (150, 125, 75, 50 MHz) spectroscopy was performed on a Bruker Avance 600, Varian Mercury 400, Varian Unity Plus 300, or Gemini 200 Unity Plus. Chemical shifts (δ) are expressed in parts per million downfield from tetramethylsilyl chloride. Coupling constants are expressed in Hertz.

Boc-Thr-Oallyl: Boc-Thr-OH (2 g, 9.8 mmol) was added to a mixture of allyl bromide (19 mL, 220 mmol) and DIEA (1.71 mL, 9.8 mmol). The mixture was heated at reflux for 4 h, cooled, filtered, diluted with EtOAc (30 mL), and finally washed with 5% $NaHCO_3$ (5 × 20 mL). Evaporation of the solvent followed by chromatography (hexane/ethyl acetate, 7:3) afforded Boc-Thr-Oallyl (2.23 g, 8.6 mmol, 93%) as a yellow oil. Analytical HPLC (t_R = 18.2 min, condition A); TLC (SiO_2): R_f = 0.44 (hexane/EtOAc 3:2); IR (film): $\tilde{\nu}$ = 3691, 3089, 2981, 1719, 1653 1368–1393, 1069 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.26 (d, J = 6.3 Hz, 3H; CH_3 Thr), 1.46 (s, 9H; 3CH_3 Boc), 2.13 (br s, 1H; OH Thr), 4.26–4.32 (m, 2H; CH^{α} Thr, CH^{β} Thr), 4.66 (m, 2H; OCH_2 allyl), 5.27 (dm, J = 10.5 Hz, 1H; =C*H*H′ allyl), 5.35 (dm, J = 17.4 Hz, 1H; =CH*H*′ allyl), 5.36 (br s, 1H; NH Thr), 5.92 ppm (m, 1H; CH allyl); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 19.9 (CH_3 Thr), 28.3 (3CH_3 Boc), 58.9 (CH^{α} Thr), 66.0 (OCH_2 allyl), 67.9 (CH^{β} Thr), 80.0 (C Boc), 118.6 (=CH_2 allyl), 131.4 (CH allyl), 156.1 (CO Boc), 171.1 ppm (CO Thr); ESMS (H_2O/CH_3CN 1:1 *v*/*v*, 1% formic acid): *m*/*z*: 204.1 [*M* – allyl+H]$^+$, 260.1 [*M*+H]$^+$, 282.1 [*M*+Na]$^+$.

HCl·H-Thr-Oallyl: A commercial solution of 4 M HCl/1,4-dioxane was added to Boc-Thr-Oallyl (1.94 g, 7.5 mmol) until this was covered and the mixture was stirred for 90 min at 25 °C. The solvent was removed in vacuo and the remaining acid and dioxane were removed by co-evaporations with Et_2O to afford the title compound (1.44 g, 7.3 mmol, 98%) as a yellow oil. TLC (SiO_2): R_f = 0.05 (hexane/EtOAc 3:2); IR (film): $\tilde{\nu}$ = 3691, 2932, 1746, 1652, 1457, 1221, 1117 cm^{-1}; ^{1}H NMR (200 MHz, CD_3OD): δ = 1.33 (d, J = 6.2 Hz; 3H, CH_3 Thr), 4.00 (d, J = 4.0 Hz, 1H; CH^{α} Thr), 4.30 (qd, J = 6.4,

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4.2 Hz, 1H; CH$^{\beta}$ Thr), 4.80 (m, 2H; OCH_2 allyl), 5.30 (dm, $J = 10.4$ Hz, 1H; =C*H*H′ allyl), 5.40 (dm, $J = 17.2$ Hz, 1H; =CH*H*′ allyl), 6.00 ppm (m, 1H; CH allyl); ^{13}C NMR (75 MHz, CD_3OD): $\delta = 18.6$ (CH_3 Thr), 57.8 (CH$^{\alpha}$ Thr), 64.3 (CH$^{\beta}$ Thr), 65.9 (OCH_2 allyl), 117.6 (=CH_2 allyl), 130.5 (CH allyl), 166.8 ppm (CO Thr); ESMS (H_2O/ACN, 1:1 v/v, 1% formic acid): *m/z*: 160.2 $[M+H]^+$.

Fmoc-Thr(*t*Bu)-OSu: A solution of Fmoc-Thr(*t*Bu)-OH (3 g, 7.5 mmol) in 1,4-dioxane (20 mL) and a solution of *N*-hydroxysuccinimide (HOSu; 1.56 g, 13.6 mmol) in 1,4-dioxane/H_2O (5.5 mL, 9:1) were mixed together and DIPCDI (1.52 mL, 9.8 mmol) was added. The mixture was stirred for 16 h at 25 °C. The solvent was evaporated in vacuo, the residue was dissolved in EtOAc, and the solution was washed with a solution of HCl (3×25 mL, pH 3). The combined organic phases were dried over $MgSO_4$, filtered, and evaporated to give Fmoc-Thr(*t*Bu)-OSu (3.55 g, 7.2 mmol, 95%) as a white solid. Analytical HPLC ($t_R = 20.2$ min, condition B); TLC (SiO_2): $R_f = 0.58$ ($CHCl_3$/MeOH 9:1); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta =$ 1.13 (d, $J = 6.4$ Hz, 3H; CH_3 Thr(*t*Bu)), 1.22 (s, 9H; 3 CH_3 *t*Bu), 2.80 (s, 4H, 2 CH_2 OSu), 4.26 (m, 1H; CH Fmoc), 4.37 (m, 1H; CH$^{\beta}$ Thr(*t*Bu)), 4.43 (m, 2H; CH_2 Fmoc), 4.62 (dd, $J = 9.2$, 2.0 Hz, 1H; CH$^{\alpha}$ Thr(*t*Bu)), 5.70 (d, $J =$ 9.6 Hz, 1H; NH Thr(*t*Bu)), 7.31 (m, 2H; 2 CH_{arom} Fmoc), 7.39 (m, 2H; 2 CH_{arom} Fmoc), 7.62 (m, 2H; 2 CH_{arom} Fmoc), 7.76 ppm (m, 2H; 2 CH_{arom} Fmoc); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 21.5$ (CH_3 Thr(*t*Bu)), 26.3 (2 CH_2 OSu),29.3 (3 CH_3 Boc), 47.9 (CH Fmoc), 59.4 (CH$^{\alpha}$ Thr(*t*Bu)), 68.0 (CH$^{\beta}$ Thr(*t*Bu)), 68.2 (CH_2 Fmoc), 75.5 (C Boc), 120.7 (2 CH_{arom} Fmoc), 125.9 (2 CH_{arom} Fmoc), 127.8 (2 CH_{arom} Fmoc), 128.5 (2 CH_{arom} Fmoc), 142.1 (2 C_{arom} Fmoc), 144.4 (2 C_{arom} Fmoc), 157.0 (CO Fmoc), 167.6 (CO Thr(*t*Bu)), 169.2 ppm (2 CO OSu); MALDI-TOF MS (DHB): *m/z*: 517.9 $[M+Na]^+$, 533.9 $[M+K]^+$.

Fmoc-Thr(*t*Bu)-Thr-Oallyl: A solution of Fmoc-Thr(*t*Bu)-OSu (2.62 g, 5.3 mmol) in 1,4-dioxane (20 mL) was added to a solution of HCl · H-Thr-Oallyl (1.44 g, 7.3 mmol) and DIEA (2.55 mL, 14.6 mmol) in 1,4-dioxane (25 mL) and the mixture was stirred for 15 h at 25 °C. The solvent was evaporated in vacuo, the residue was taken up in EtOAc (40 mL), and the solution washed with brine (2×25 mL) and aqueous HCl (pH 3–4, $3 \times$ 25 mL). The combined organic phases were dried over $MgSO_4$, filtered, and evaporated in vacuo to dryness to provide the title dipeptide (2.43 g, 4.5 mmol, 85%) as a white solid; Analytical HPLC ($t_R = 21.2$ min, condition B); TLC (SiO_2): $R_f = 0.54$ (CH_2Cl_2/acetone 9:1); ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 1.10$ (d, $J = 6.6$ Hz, 3H; CH_3 Thr(*t*Bu)), 1.23 (d, $J = 6.6$ Hz, 3H; CH_3 Thr), 1.29 (s, 9H; 3 CH_3 *t*Bu), 2.04 (d, $J = 3.6$ Hz, 1H; OH Thr), 4.18 (m, 1H, CH$^{\beta}$ Thr(*t*Bu)), 4.22 (m, 1H; CH Fmoc), 4.28 (m, 1H; CH$^{\alpha}$ Thr(*t*Bu)), 4.40 (m, 3H; CH_2 Fmoc, CH$^{\beta}$ Thr), 4.55 (dd, $J = 8.4$, 2.4 Hz, 1H; CH$^{\alpha}$ Thr), 4.66 (m, 2H; OCH_2 allyl), 5.25 (dm, $J = 10.2$ Hz, 1H; =C*H*H′ allyl), 5.34 (dm, $J = 17.4$ Hz, 1H; =CH*H*′ allyl), 5.90 (m, 1H; CH allyl), 6.00 (d, $J = 5.4$ Hz, 1H; NH Thr(*t*Bu)), 7.29 (m, 2H; 2 CH_{arom} Fmoc), 7.38 (m, 2H; 2 CH_{arom} Fmoc), 7.59 (m, 2H; 2 CH_{arom} Fmoc), 7.74 (m, 2H; 2 CH_{arom} Fmoc), 8.05 ppm (d, $J = 8.4$ Hz, 1H; NH Thr); ^{13}C NMR (150 MHz, $CDCl_3$): $\delta = 16.6$ (CH_3 Thr(*t*Bu)), 20.3 (CH_3 Thr), 28.4 (3 CH_3 *t*Bu), 47.7 (CH Fmoc), 57.9 (CH$^{\alpha}$ Thr), 58.9 (CH$^{\alpha}$ Thr(*t*Bu)), 66.3 (OCH_2 allyl), 67.2 (CH$^{\beta}$ Thr(*t*Bu)), 67.4 (CH$^{\beta}$ Thr, CH_2 Fmoc), 75.9 (C *t*Bu), 119.3 (=CH_2 allyl), 120.4 (2 CH_{arom} Fmoc), 125.5 (2 CH_{arom} Fmoc), 127.4 (2 CH_{arom} Fmoc), 128.0 (2 CH_{arom} Fmoc), 131.8 (CH allyl), 141.6 (2 C_{arom} Fmoc), 144.2 (2 C_{arom} Fmoc), 156.9 (CO Fmoc), 170.0 ppm (CO Thr(*t*Bu), CO Thr); MALDI-TOF MS (DHB): *m/z*: 562.0 $[M+Na]^+$, 577.9 $[M+K]^+$.

Fmoc-Thr(*t*Bu)-(*Z*)-Dhb-Oallyl: Fmoc-Thr(*t*Bu)-Thr-Oallyl (2.43 g, 4.5 mmol), EDC · HCl (1.73 g, 9.0 mmol), and CuCl (0.54 g, 5.42 mmol) were dissolved in CH_2Cl_2/DMF (98:2) (40 mL) under N_2 and the mixture was stirred for 20 h at 25 °C. The organic solvent was evaporated in vacuo and the residue was purified by flash chromatography (CH_2Cl_2/acetone, from 1:0 to 9:1) to give the title compound (1.6 g, 3.1 mmol, 70%). Analytical HPLC ($t_R = 23.6$ min, condition B); TLC (SiO_2): $R_f = 0.47$ (CH_2Cl_2/acetone 98:2); ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 1.12$ (d, $J =$ 6.6 Hz, 3H; CH_3 Thr(*t*Bu)), 1.30 (s, 9H; 3 CH_3 *t*Bu), 1.78 (d, $J = 7.2$ Hz, 3H; CH_3 Dhb), 4.22 (m, 2H; CH Fmoc, CH$^{\beta}$ Thr(*t*Bu)), 4.32 (dd, $J = 4.8$, 4.8 Hz, 1H; CH$^{\alpha}$ Thr(*t*Bu)), 4.40 (m, 2H; CH_2 Fmoc), 4.68 (m, 2H; OCH_2 allyl), 5.25 (dm, $J = 10.2$ Hz, 1H; =C*H*H′ allyl), 5.34 (dm, $J = 17.4$ Hz, 1H, =CH*H*′ allyl), 5.93 (m, 1H, CH allyl), 6.00 (d, $J = 4.8$ Hz, 1H; NH Thr(*t*Bu)), 6.76 (q, $J = 7.2$ Hz, 1H; CH Dhb), 7.31 (m, 2H; 2 CH_{arom} Fmoc), 7.39 (m, 2H; 2 CH_{arom} Fmoc), 7.62 (m, 2H; 2 CH_{arom} Fmoc), 7.76 (m, 2H; 2 CH_{arom} Fmoc), 8.58 ppm (s, 1H; NH Dhb); ^{13}C NMR (150 MHz, $CDCl_3$): $\delta = 15.7$ (CH_3 Dhb), 17.2 (CH_3 Thr(*t*Bu)), 28.3 (3 CH_3 *t*Bu), 47.5 (CH Fmoc), 59.4 (CH$^{\alpha}$ Thr(*t*Bu)), 66.1 (OCH_2 allyl), 66.9 (CH$^{\beta}$ Thr (*t*Bu)), 67.3 (CH_2 Fmoc), 76.3 (C *t*Bu), 118.8 (=CH_2 allyl), 120.5 (2 CH_{arom} Fmoc), 125.6 (2 CH_{arom} Fmoc), 127.4 (2 CH_{arom} Fmoc), 128.0 (2 CH_{arom} Fmoc), 126.9 (C Dhb), 127.6 (CH allyl), 133.2 (CH Dhb), 141.8 (2 C_{arom} Fmoc), 144.1 (2 C_{arom} Fmoc), 156.5 (CO Fmoc), 164.2 (CO Dhb), 168.2 ppm (CO Thr(*t*Bu)); MALDI-TOF MS (DHB): *m/z*: 543.8 $[M+Na]^+$, 559.8 $[M+K]^+$.

Fmoc-Thr(*t*Bu)-(*Z*)-Dhb-OH: Fmoc-Thr(*t*Bu)-(*Z*)-Dhb-Oallyl (1.6 g, 3.1 mmol) and $PhSiH_3$ (1.14 mL, 9.2 mmol) were dissolved in CH_2Cl_2 (20 mL) and the mixture was purged with Ar before adding $[Pd(PPh_3)_4]$ (107 mg, 0.1 mmol). The mixture was purged again and stirred under argon for 2 h at 25 °C. H_2O (277 μL, 15.38 mmol) was added and the mixture was filtered. The solvent was evaporated in vacuo and the residue was purified by flash chromatography (CH_2Cl_2/acetone/MeOH 90:8:2) to provide the final dipeptide (614 mg, 42%) as a yellow solid. Analytical HPLC ($t_R =$ 19.7 min, condition B); TLC (SiO_2): $R_f = 0.24$ (CH_2Cl_2/acetone/MeOH, 90:8:2); ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 1.12$ (d, $J = 6.0$ Hz, 3H; CH_3 Thr(*t*Bu)), 1.30 (s, 9H; 3 CH_3 *t*Bu), 1.81 (d, $J = 7.2$ Hz, 3H; CH_3 Dhb), 4.22 (m, 1H; CH Fmoc), 4.24 (m, 1H; CH$^{\beta}$ Thr(*t*Bu)), 4.33 (m, 1H, CH$^{\alpha}$ Thr(*t*Bu)), 4.40 (m, 2H; CH_2 Fmoc), 6.00 (d, $J = 5.4$ Hz, 1H; NH Thr(*t*Bu)), 6.87 (q, $J = 7.2$ Hz, 1H; CH Dhb), 7.31 (m, 2H; 2 CH_{arom} Fmoc), 7.39 (m, 2H; 2 CH_{arom} Fmoc), 7.62 (m, 2H; 2 CH_{arom} Fmoc), 7.76 (m, 2H; 2 CH_{arom} Fmoc), 8.58 ppm (s, 1H; NH Dhb); ^{13}C NMR (150 MHz, $CDCl_3$): $\delta = 15.2$ (CH_3 Dhb), 23.7 (CH_3 Thr(*t*Bu)), 28.3 (CH_3 *t*Bu), 47.8 (CH Fmoc), 59.3 (CH$^{\alpha}$ Thr(*t*Bu)), 67.1 (CH$^{\beta}$ Thr(*t*Bu), 67.2 (CH_2 Fmoc), 120.6 (2 CH_{arom} Fmoc), 125.8 (2 CH_{arom} Fmoc), 127.8 (2C H_{arom} Fmoc), 128.4 (2 CH_{arom} Fmoc), 135.1 (C Dhb), 141.3 (2 C_{arom} Fmoc), 143.7 (2 C_{arom} Fmoc), 156.3 (CO Fmoc), 167.6, 168.2 ppm (CO Thr(*t*Bu), CO Dhb); MALDI-TOF MS (DHB): *m/z*: 503.8 $[M+Na]^+$, 519.7 $[M+K]^+$.

Alloc-Ser-OH: H-L-Ser-OH (1.2 g, 9.5 mmol) was suspended in CH_2Cl_2 (10 mL) under an argon atmosphere and stirred vigorously. TMSCl (3.1 g, 28.5 mmol) was added in one portion and the mixture was heated at reflux for 2 h and then cooled in an ice bath for 30 min; a white precipitate formed. DIEA (4.70 mL, 27.0 mmol) was added to the mixture and allyl chloroformate (0.84 mL, 7.9 mmol) was added dropwise. The cooled (ice bath) mixture was stirred for 20 min and then warmed up to 25 °C for 2 h. The solvent was evaporated in vacuo. The residue was dissolved in 10% aqueous $NaHCO_3$, washed with EtOAc (3×40 mL), and the combined aqueous layers were acidified to pH 2 with 1M HCl and extracted again with EtOAc (3×50 mL). The latter EtOAc layers were dried over $MgSO_4$, filtered, and evaporated in vacuo to give Alloc-Ser-OH (1.05 g, 5.6 mmol, 70%) as a yellow solid. Analytical HPLC ($t_R = 10.1$ min, condition A); IR (film) $\tilde{\nu} = 3670$, 3087, 2950, 1703 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta =$ 3.98 (m, 2H, CH_2 Ser), 4.41 (m, 1H, CH Ser), 4.59 (m, 2H, CH_2O Alloc), 5.22 (dm, $J = 10$ Hz, 1H, =C*H*H′), 5.31 (dm, $J = 18$ Hz, 1H, =CH*H*′), 5.47 (br s, 1H; OH Ser), 5.91 (m, 1H; CH Alloc), 6.10 ppm (m, 1H; NH Ser); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 55.8$ (CH Ser), 62.7 (CH_2 Ser), 66.2 (CH_2O Alloc), 117.9 (CH_2= Alloc), 132.3 (CH Alloc), 156.6 (CO Alloc), 173.5 ppm (CO Ser); MALDI-TOF MS (DHB): *m/z*: 212.4 $[M+Na]^+$, 228.4 $[M+K]^+$.

Mst-Ser-[*O*-Thr(*t*Bu)-Asp(O*t*Bu)-(*Z*)-Dhb-Thr(*t*Bu)-Orn(Boc)-D-Ser-H]-D-Dab(Boc)-Gly-OH: Barlos resin (150 mg, 1.6 mmol g^{-1}) was placed in a 10 mL polypropylene syringe fitted with a polyethylene filter disc. The resin was then washed with CH_2Cl_2 (5×1 min), DMF (5×1 min), and CH_2Cl_2 (5×1 min). A solution of Fmoc-Gly-OH (31 mg, 0.106 mmol) and DIEA (93 μL, 0.53 mmol, 0.66 equiv) in CH_2Cl_2 (2.5 mL) was then added. After 5 min, DIEA (186 μL, 1.06 mmol, 1.33 equiv) was added and the mixture was stirred for 40 min at 25 °C. The reaction was terminated by the addition of MeOH (0.15 mL) and the mixture was stirred for a further 10 min. The Fmoc-Gly-*O*-Barlos resin was subjected to the following washings/treatments: CH_2Cl_2 (5×0.5 min), DMF (5×0.5 min), piperidine/DMF (2:8) (1×1 min, 3×5 min, 1×10 min), DMF (5×1 min), MeOH (2×1 min), CH_2Cl_2 (3×1 min). The loading, as calculated by amino acid analysis, was 0.59 mmol g^{-1}.

Fmoc-D-Dab(Boc)-OH (99.1 mg, 3 equiv) was coupled to the Barlos resin using DIPCDI (34.8 μL, 3 equiv) and HOBt (34.5 mg, 3 equiv) in DMF. The ninhydrin test was negative after two couplings of 1 h each. The Fmoc group was removed as described above and Alloc-Ser-OH (42 mg, 3 equiv) was incorporated by treatment with TBTU (72.2 mg, 3 equiv), HOBt (34.4 mg, 3 equiv), and DIEA (78.4 μL, 6 equiv) for 1 h at 25 °C. Fmoc-Thr(*t*Bu)-OH (149.1 mg, 5 equiv) and DIPCDI (58 μL, 5 equiv) were dissolved in DMF and the solution was added to the peptide resin, followed by addition of DMAP (4.77 mg, 0.5 equiv) to provide the ester linkage. The

mixture was stirred for 30 min and the treatment was repeated twice. The alcohol test was negative.[24]

Removal of the Alloc group was carried out with three treatments of $[Pd(PPh_3)_4]$ (8.7 mg, 0.1 equiv) and $PhSiH_3$ (92.4 μL, 10 equiv) dissolved in CH_2Cl_2 for 15 min at 25 °C under Ar. Mst (85.6 mg, 5 equiv) was coupled to the H-Ser-[*O*-Thr(*t*Bu)-Fmoc]-D-Dab(Boc)-Gly-*O*-Barlos resin by using DIPCDI (58 μL, 5 equiv) and HOBt (57.4 mg, 5 equiv) in DMF. The ninhydrin test was negative after two couplings of 1 h each.

After removal of the Fmoc group, Fmoc-Asp(O*t*Bu)-OH (154 mg, 5 equiv) was coupled to the Mst-Ser-[*O*-Thr(*t*Bu)-H]-D-Dab(Boc)-Gly-Barlos resin as described previously for Mst.

The Fmoc group was removed as described previously and Fmoc-Thr(*t*Bu)-(*Z*)-Dhb-OH (108 mg, 3 equiv) was incorporated with DIPCDI (35 μL, 3 equiv) and HOAt (35 mg, 3 equiv) in DMF. After stirring for 3 h, more DIPCDI (18 μL, 1.5 equiv) and HOAt (17 mg, 1.5 equiv) were added. The ninhydrin test was negative after coupling overnight.

Fmoc-Orn(Boc)-OH (170 mg, 5 equiv) and Fmoc-D-Ser-OH (122.7 mg, 5 equiv) were coupled sequentially to the peptide resin using DIPCDI (58 μL, 5 equiv) and HOBt (57.4 mg, 5 equiv) in DMF. In both cases, the ninhydrin test was negative after a second coupling.

After removal the Fmoc group, the peptide was cleaved from the resin with TFA/CH_2Cl_2 (1:99) (5×30 s), collecting the filtrates over H_2O (10 mL, 60 mL $H_2O\,g^{-1}$ resin). The combined filtrates were evaporated to dryness under reduced pressure to give title compound (57 mg, 35.9 μmol) with a purity of >61% as determined by HPLC (condition C). MALDI-TOF MS (DHB): *m/z*: 1465.0 $[M+Na]^+$, 1481.0 $[M+K]^+$.

Mst-cyclo[Ser-D-Dab(Boc)-Gly-D-Ser-L-Orn(Boc)-L-Thr(*t*Bu)-(*Z*)-Dhb-L-Asp(O*t*Bu)-Thr(*t*Bu)]: An aliquot (25 mg, 17.3 μmol) of the crude linear peptide dissolved in CH_2Cl_2 (17 mL) was added to a solution of HOBt (10.6 mg, 4 equiv) in the minimum amount of DMF (50 μL). DIEA (9.1 μL, 3 equiv) and DIPCDI (10.7 μL, 4 equiv) were then added, and the mixture was stirred for 1.5 h at 25 °C. The solvent was removed in vacuo, H_2O was added and lyophilizated, and the crude product had a purity of 44% by analytical HPLC ($t_R = 23.3$ min, condition D). The crude product was purified by MPLC (Vydac C_8 10 μm, 300 Å, 250×10 mm) by using a linear gradient from 40 to 80% acetonitrile (+0.1% TFA) in water (+0.1% TFA) over 2 h (300 mL each solvent) at a flow rate of 120 mL h^{-1} with detection at 220 nm. Analytical HPLC ($t_R = 23.3$ min, condition D, 90% purity); MALDI-TOF MS (DHB): *m/z*: 1448.5 $[M+Na]^+$, 1465.5 $[M+K]^+$.

[*N*-Mst(Ser1), D-Ser4, L-Thr6, L-Asp8, L-Thr9]syringotoxin: The pure cyclic peptide was dissolved in TFA/H_2O (19:1) (10 mL) and vigorously stirred for 1.5 h at 25 °C. The TFA was evaporated under reduced pressure and the remaining acid was removed by co-evaporations with Et_2O. The residue was triturated with Et_2O, the solution was decanted, and the operation was repeated three times to afford the target compound (2.5 mg, 14% overall yield for cyclization, purification and deprotection step). Analytical HPLC ($t_R = 12.8$ min, condition E, >90% purity); MALDI-TOF MS (DHB): *m/z*: 1057.7 $[M+H]^+$, 1079.7 $[M+Na]^+$.

Biological activity: Leishmanicidal activity was tested on *Leishmania donovani* promastigotes as described in the literature.[35] Briefly, parasites were resuspended at a concentration of 2×10^7 cells mL^{-1} in phosphate saline buffer and incubated with the peptides for 4 h at 25 °C. Surviving parasites were allowed to proliferate for 72 h. LD_{50} values were calculated by the Lichfield and Wilcoxon procedure and the 95% confidence interval is given in parentheses.

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[15] Attempts to prepare Fmoc-Thr(*t*Bu)-Thr-Oallyl from Fmoc-Thr(*t*Bu)-OH and HCl·H-Thr-Oallyl by using carbodiimide-based methods (EDC·HCl/DIEA, DIPCDI/DIEA, DIPCDI/HOBt/DIEA) led to incomplete dipeptide formation, presumably due to some degree of steric hindrance between the two β-branched amino acids.
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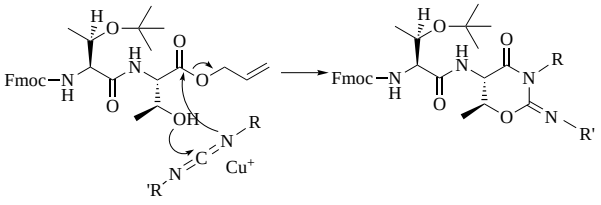

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