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Synthesis and biological evaluation of potential threonine synthase inhibitors: Rhizocticin A and Plumbemycin A

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ABSTRACT

Rhizocticins and Plumbemycins are natural phosphonate antibiotics produced by the bacterial strains *Bacillus subtilis ATCC 6633* and *Streptomyces plumbeus*, respectively. Up to now, these potential threonine synthase inhibitors have only been synthesized under enzymatic catalysis. Here we report the chemical stereoselective synthesis of the non-proteinogenic (*S*,*Z*)-2-amino-5-phosphonopent-3-enoic acid [(*S*,*Z*)-APPA] and its use for the synthesis of Rhizocticin A and Plumbemycin A. In this work, (*S*,*Z*)-APPA was synthesized via the Still–Gennari olefination starting from Garner's aldehyde. The Michaelis–Arbuzov reaction was used to form the phosphorus–carbon bond. Oligopeptides were prepared using liquid phase peptide synthesis (LPPS) and were tested against selected bacteria and fungi.

1. Introduction

Today some strains of bacteria are resistant to all commercially available antibiotics and the search for new drugs with alternative cellular targets is highly needed. In this context, we have started research on the phosphonate antibiotics. Phosphonates represent an important class of organophosphorus compounds characterized by the presence of one or more carbon-to-phosphorus (C–P) bonds. Ciliatine (2-aminoethylphosphonic acid—AEP) was the first phosphonate discovered in 1959 by Kandatsu and Horigushi from hydrolysates of ciliated protozoa.[1] From this date, many other phosphonates were gradually discovered or synthesized. Currently, these compounds attracted the attention of researchers for their use as reagents in organic synthesis, as herbicides or pesticides in agriculture, as chemical warfare and most of all, for their interesting medical properties. Indeed, we find among these phosphonates; antibiotics,[2–4] antivirals,[5] antitumor compounds,[6] antimalarial agents,[7,8] antihypertensives,[9] and many other. The biological activity of some phosphonates stems from their structural similarity to phosphate esters. Other members of this class mimic carboxyl groups or the tetrahedral intermediates formed during enzymatic metabolism.[10] In this work, we develop a chemical synthesis of naturally occurring phosphonate antibiotics; Rhizocticins (RZs, Fig. 1A) and Plumbemycins (PBs, Fig. 1B).

The structure of Rhizocticins (RZs) antibiotics was elucidated by Rapp et al.[11] and by Fredenhagen et al. using RZs isolated from culture media.[12] Four different RZs have been isolated from a complex culture media of *Bacillus subtilis* ATCC 6633: RZ-A = (*S*)-Arg-(*S*,*Z*)-APPA, RZ-B = (*S*)-Val-(*S*)-Arg-(*S*,*Z*)-APPA, RZ-C = (*S*)-Ile-(*S*)-Arg-(*S*,*Z*)-APPA and RZ-D = (*S*)-Leu-(*S*)-Arg-(*S*,*Z*)-APPA (Fig. 1**A**). It is interesting to note that (*S*,*Z*)-APPA is the C-terminal component of two other antibiotics: Plumbemycin A (PB-A) and Plumbemycin B (PB-B), (Fig. 1B) isolated in 1976 from *Streptomyces plumbeus* by Park, Hirota and Sakai.[13] PB-A = (*S*)-Ala-(*S*)-Asp-(*S*,*Z*)-APPA and PB-B = (*S*)-Ala-(*S*)-Asn-(*S*,*Z*)-APPA. The *Z*-APPA moiety of PBs was first reported to be in a (*R*)-configuration based on the *Clough–Lutz–Jirgensons* (*CLJ*) rule.[14] However, this observation was later contested by Fredenhagen et al.[12] who demonstrated by chiral HPLC analysis and optical rotation measurement that (*Z*)-APPA has (*S*)-configuration both in RZs and PBs. RZs are antifungal whereas PBs are antibacterial. The selectivity of these compounds is due to the recognition of amino acids attached to the (*S*,*Z*)-APPA moiety by specific oligopeptide transport system.[15] RZs and PBs are hydrolyzed by oligopeptidases into the active (*S*,*Z*)-APPA moiety and inactive amino acids.

The target of the (*S*,*Z*)-APPA moiety of these antibiotics in bacteria and fungi is threonine synthase (TS),[16] the last enzyme

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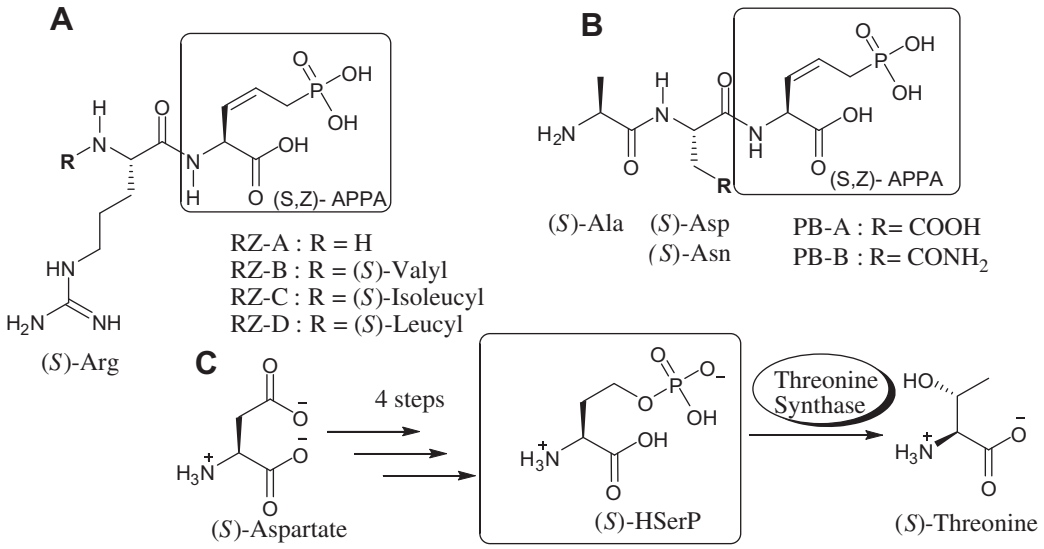


Figure 1. Chemical structures of (*S,Z*)-APPA and its containing oligopeptide phosphonate antibiotics (A) = Rhizocticins, (B) = Plumbemycins. (C) = threonine biosynthetic pathway from (*S*)-aspartate.

of threonine biosynthetic pathway, which catalyses the formation of (*S*)-threonine from the substrate (*S*)-homoserine-γ-phosphate, (*S*)-HSerP (Fig. 1C). Since TS is absent in mammals, enzymes of this pathway are potential targets for the development of new antibiotics.[17,18] In this work, we first present the stereoselective chemical synthesis and characterization of the non-proteinogenic (*S,Z*)-APPA **8** as an analogue of (*S*)-homoserine-γ-phosphate. Secondly, we develop the synthesis of (*S,Z*)-APPA containing phosphonate antibiotics (RZ-A and PB-A) in LPPS. Finally their biological evaluation is done.

2. Chemical results and discussion

2.1. Synthesis of (*S,Z*)-2-amino-5-phosphono-3-pentenoic acid: (*S,Z*)-APPA 8

The natural (*S,Z*)-APPA **8** was isolated and characterized for the first time by Park et al. as a constituent of PBs.[13] In 1988, an attempt for a chemical synthesis of this compound in order to assemble the antibiotics PB-A and B was done by Natchev.[19] However, according to Fredenhagen's observation,[12] and based on the starting material (*E*-4-bromocrotonaldehyde) used in this synthesis, Natchev has probably synthesized the *trans* isomer (*E*)-APPA in place of the required (*Z*)-APPA.

Here, in order to overcome this stereoselectivity problem, we planned to synthesize (*S,Z*)-APPA **8** in seven steps as shown in (Scheme 1). The first key step was the stereo- and enantioselective synthesis of the (*S,Z*)-**2** ester starting from (*R*)-Garner's aldehyde **1**[20] and Still–Gennari reagent[21] by following the protocol previously published by Shimamoto and Ohfune.[22] Therefore, $(CF_3CH_2O)_2P(=O)CH_2CO_2Me$ was reacted with NaH at 0 °C in THF in order to form the corresponding phosphonate anion in situ. To this solution cooled at −78 °C was added 18-Crown-6 (5 equiv) and (*R*)-aldehyde **1** (1 equiv) in THF at −78 °C.

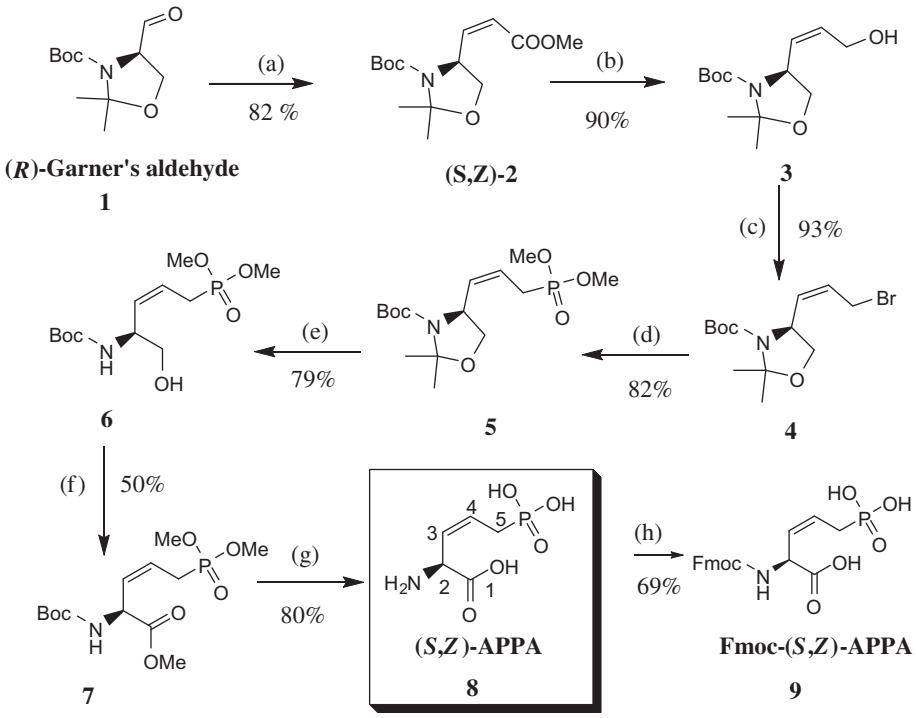
After standard work up, the crude product was analyzed by ^{1}H NMR and according to the olefin proton integration, a mixture of (*S,Z*)-**2** ester and its (*S,E*) isomer was obtained with high *Z*-selectivity (Z/*E* >95/5). The two stereo isomers were separated by silica gel column chromatography and pure (*S,Z*)-**2** was obtained as a white solid with good yield (82%). Finally, the structure of (*S,Z*)-**2** and its (*E*)-isomer was confirmed by 1D NMR (^{1}H, ^{13}C, DEPT) and 2D NMR (^{1}H–^{1}H COSY, ^{1}H–^{13}C HSQC). Importantly, the (*Z*)-geometry of (*S,Z*)-**2** was confirmed by the value of the coupling constant of the doublet (d, $^3J_{H-H}$ = 11.4 Hz) corresponding to the olefinic proton CH=C*H*–COOMe at 5.78 ppm.

The (*R,Z*)-enantiomer of (*S,Z*)-**2** was synthesized as above from (*S*)-Garner's aldehyde for comparative enantiomeric excess determination by chiral HPLC. In the HPLC chromatogram, the *R*-enantiomer eluted before the *S*-enantiomer (t_R = 7.8 min and 8.5 min, respectively). The ee found for (*S,Z*)-**2** was 94% and its optical rotation was measured as $[\alpha]_D^{25}$ −27.3 (*c* = 0.91, $CHCl_3$). Lit[22], $[\alpha]_D^{23}$ −32.6; (*c* = 1.4, $CHCl_3$). For the (*R,Z*) enantiomer , ee = 96% and the measured $[\alpha]_D^{25}$ +28.5 (*c* = 1.03, $CHCl_3$).

Having accomplish the stereo and enantioselective synthesis of compound (*S,Z*)-**2**, the second step was the chemoselective reduction of its ester function to produce the corresponding (*S,Z*)-allyl alcohol **3** using 3 equiv of DIBAL-H solution at −78 °C.[22] Under these conditions, **3** was obtained as a colorless oil with an excellent yield (90%) without the need of a chromatographic purification.

At the third step, (*S,Z*)-allyl alcohol **3** was converted to the corresponding (*S,Z*)-allyl bromide **4**. Therefore, the alcohol **3** was converted into the corresponding mesylate using MsCl and Et_3N in CH_2Cl_2 and in *one flask* manner, the mesylate was subjected in a SN_2 displacement using LiBr[23] to furnish allyl bromide **4** as a colorless oil. However, with our substrate, this reaction was not reproducible and only moderated yields were obtained (60–70%). In order to increase the yield of the reaction, we next tried the Appel reaction by reacting allyl alcohol **3** with CBr_4 (1.3 equiv) and PPh_3 (1.5 equiv) in CH_2Cl_2 at 0 °C.[24] Under these conditions, (*S,Z*)-allyl bromide **4** was obtained as a colorless oil after a simple flash chromatography, with an excellent yield (93%).

The second key step to obtain (*S,Z*)-APPA **8** was the formation of the phosphorous carbon bond using the classical Michaelis–Arbuzov reaction[25] by heating (*S,Z*)-allyl bromide **4** in an excess of trimethylphosphite $P(OMe)_3$ at 80–100 °C. Under these conditions, the reaction was complete after 6 h. The excess of $P(OMe)_3$ was evaporated and the crude product purified by flash chromatography to yield the oxazolidine phosphonate **5** in 82%. The expected mass of phosphonate **5** was confirmed by HRMS. The 1D and 2D NMR spectra confirmed also its structure (Supplementary data). It is important to note that in the ^{1}H decoupled ^{31}P NMR spectrum, two peaks



Scheme 1. Seven steps synthesis of (*S*,*Z*)-APPA **8** starting with (*R*)-Garner's aldehyde. Reagents and conditions: (a) $(CF_3CH_2O)_2P(=O)CH_2CO_2Me$, 18-Crown-6, NaH, THF, −78 °C; (b) DIBAL-H 1 M sol. in hexanes, toluene, −78 °C; (c) CBr_4, PPh_3, CH_2Cl_2, 0 °C; (d) $P(OMe)_3$, 80–100 °C; (e) Dowex 50WX4 H^+, MeOH/H_2O:(9/1); (f) (i) H_5IO_6/CrO_3, CH_3CN/H_2O:(99/1), 0 °C; (ii) CH_3I, K_2CO_3, CH_3CN; (g) (i) 10% $(CH_3)_3SiBr$ in CH_2Cl_2, and then MeOH/H_2O:(9/1); (ii) 1 N LiOH, THF/H_2O:(4/1); (h) Fmoc-OSu, $NaHCO_3$, H_2O/acetone:(50/50).

with the same intensity were observed at 30.08 and 29.29 ppm because of the presence of two rotamers. After characterization, the synthesis of **5** was scaled up to grams.

As shown in Scheme 1, the fifth step was the opening of the oxazolidine ring of phosphonate **5** to provide the alcohol **6** without removal of the N-Boc-protective group. Thus, Dowex 50WX4 H^+ resin was used to achieve this selective ring opening in MeOH/H_2O:(9/1)[26] and under these conditions Boc-phosphono amino alcohol **6** was obtained as a colorless oil after column chromatography (79% yield). The structure of **6** was confirmed by 1D and 2D NMR spectra. We observed that the ^1H decoupled ^{31}P NMR spectrum of **6** gave a single peak at 30.14 ppm. This indicated that the phenomenon of rotamers was suppressed with the opening of the oxazolidine ring.

The alcohol **6** was then oxidized to the corresponding acid using Zhao's reagent.[27,28] First, we tried a deprotection of the crude acid using 10% $(CH_3)_3SiBr$ in CH_2Cl_2 in order to form the target molecule (*S*,*Z*)-APPA **8** directly. However, under these conditions, a red complex mixture was obtained, probably because of the interaction between $(CH_3)_3SiBr$ and the free carboxylic acid function. To overcome this problem, we decided to convert the crude acid into the corresponding methyl ester **7** using excess of CH_3I in the presence of K_2CO_3; before deprotection. Pure methyl ester **7** was obtained in yield of 50% as colorless oil after column chromatography and was characterized by 1D and 2D NMR. The ^1H decoupled ^{31}P NMR spectrum of **7** gave a single peak at 28.99 ppm.

Finally, a deprotection of ester **7** was performed in two steps. First, the simultaneous cleavage of the phosphonate methyl group and the Boc protective group was achieved with Me_3SiBr (10% in CH_2Cl_2).[29] Secondly, the methyl ester was saponified by LiOH. The excess of LiOH was neutralized by Dowex 50WX4 H^+ and after filtration of the resin, the product was purified by several washing steps of its aqueous solution with CH_2Cl_2. We obtained the target (*S*,*Z*)-APPA **8** as a white solid in good yield (80%) for the two steps of deprotection followed by lyophilization.

The expected mass of (*S*,*Z*)-APPA **8** was confirmed by LRMS and HRMS. The ^1H and ^{13}C NMR spectra assignments were in agreement with those of *Z*-APPA isolated by acidic hydrolysis of natural PBs[13] or RZs[11,12] and were different from the ^1H and ^{13}C NMR spectra of its *trans* isomer (*S*,*E*)-APPA published in the literature.[30,31]

The ^1H NMR and COSY ^1H–^1H spectra of **8** in D_2O showed the olefinic protons at 6.04–5.93 ppm (m, 1H) for H-4 and at 5.64 ppm (td, $^3J_{H-H}$ = 10.3 Hz) for H-3, respectively. This value of coupling constant between the olefinic protons of **8** indicated a (*Z*) configuration. The H-2 signal appears at 4.75 ppm (d, $^3J_{H-H}$ = 10.2 Hz) and the typical signal corresponding to CH_2-P was a broad multiplet that extends from 2.82 to 2.55 ppm. The ^1H decoupled ^{31}P NMR spectrum of **8** revealed a single signal at 19.20 ppm and a transformation of this signal to a triplet of triplet in the ^1H-coupled ^{31}P NMR spectrum was observed. The ^{31}P–^1H HSQC spectrum confirmed the simultaneous coupling of the phosphorus and H-3, H-4 and H-5 protons. This explains why the signal was a triplet of triplet. Importantly, the typical large coupling constant $^1J_{C-P}$ = 127.0 Hz at 28.38 ppm confirmed the CH_2–P bond.[11,13] It is important to note that in the NMR data, published by Park et al.[13] and by Rapp et al.[11], for *Z*-APPA obtained by acidic hydrolysis of PBs and RZs, both purified from bacterial cultures, the same value ($^1J_{C-P}$ = 127.0 Hz) was obtained in the ^{13}C NMR spectrum. Furthermore, the ^{13}C, the HSQC and the DEPT NMR spectra of **8** in D_2O support the structure shown in Scheme 1. Unfortunately, the $^1J_{C-P}$ coupling constant for *E*-APPA isomer is not published. Thus, comparison of Z-APPA **8** with its *E*-APPA isomer based on the value of $^1J_{C-P}$ was not possible.

We have solved the problem of detecting the (*S*,*Z*)-APPA **8** in the conventional UV coupled HPLC by protecting its amine function

with the Fmoc chromophore to produce Fmoc-(*S*,*Z*)-APPA **9** (69% yield) for enantiomeric excess determination. For comparison, the Fmoc-(*R*,*Z*)-APPA enantiomer of **9** was also synthesized from (*S*)-Garner's aldehyde following the same seven steps as in Scheme 1. Thus, chiral HPLC of the two enantiomers was done using Astec Chirobiotic T column. In the HPLC chromatogram, the Fmoc-(*S*,*Z*)-APPA eluted before the Fmoc-(*R*,*Z*)-APPA enantiomer and the enantiomeric excess of Fmoc-(*S*,*Z*)-APPA **9** was ee = 90%. Finally, the measured optical rotation of Fmoc-(*S*,*Z*)-APPA **9** was $[\alpha]_D^{25}$ −15.9 (*c* = 0.46, EtOAc).

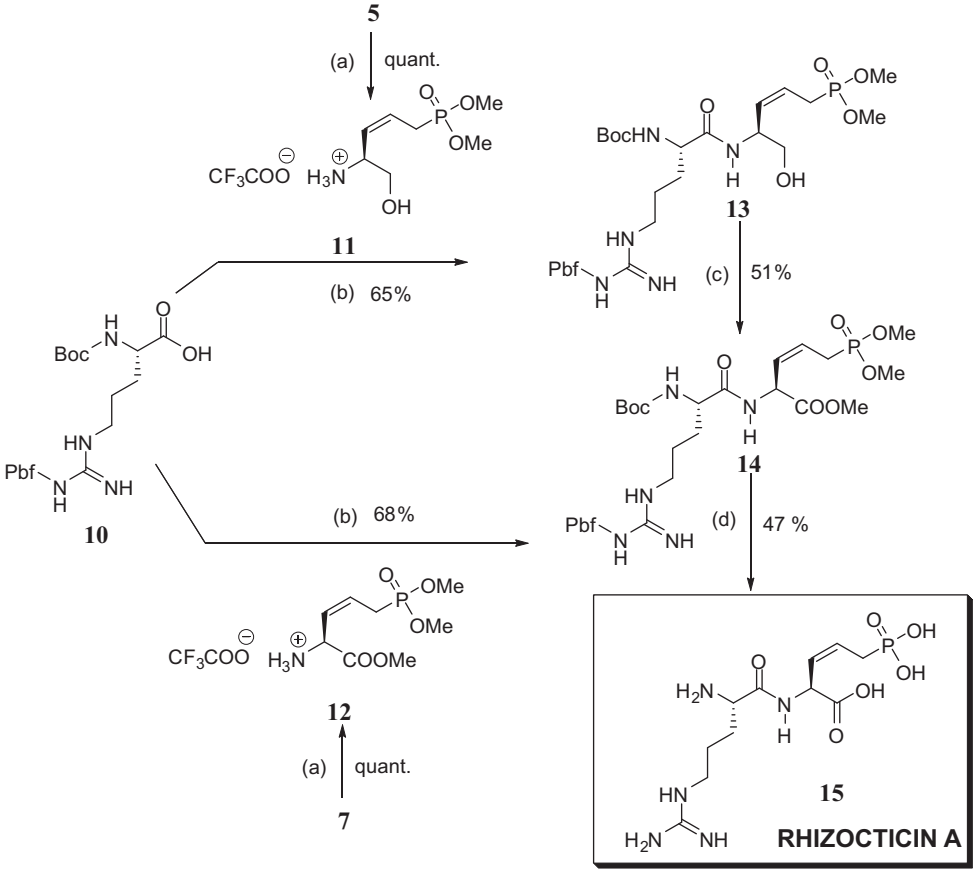
2.2. Synthesis of Rhizocticin A: RZ-A 15

Scheme 2 shows the peptide coupling reactions used to obtain RZ-A **15** = (*S*)-Arg-(*S*,*Z*)-APPA. Arylsulfonyl protecting groups are the best blocking groups for guanidine side chain of arginine. Their relative acid lability increases in this order: Tos < Mts < Mtr < Pmc < Pbf < MIS.[32] Today, the Pbf protective group displays the best deprotection kinetics among the Arg derivatives commercially available. For this reason, commercially obtained Boc-(*S*)-Arg(Pbf)-OH **10** was used to form phosphono-oligopeptides **13** and **14** in liquid phase. The advantage of this compound is that the Boc and Pbf protective groups could be removed simultaneously by TFA solutions at the step of deprotection.[33]

To obtain fully protected dipeptide **14**, two approaches were followed. In the first approach, phosphono-aminoalcohol **11** obtained after TFA deprotection of compound **5** was used. The coupling reagent *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(benzotriazol-1-yl) uronium tetrafluoroborate (TBTU)[34,35] or 3-(Diethoxyphos-phoryloxy)-1,2,3-benzotriazin-4(3*H*) one (DEPBT), a coupling agent for peptidol synthesis[36] were used. Thus, the reaction was made in THF/DMF between Boc-(*S*)-Arg(Pbf)-OH **10** and freshly deprotected **11** in presence of TBTU or DEPBT as coupling agents and $KHCO_3$ as base. After preparative HPLC purification, the protected dipeptidol **13** was obtained with a yield of 65% for TBTU and 50% for DEPBT coupling reagents.

Oxidation of **13** to the corresponding carboxylic acid using H_5IO_6/CrO_3 and subsequent esterification of the crude acid with CH_3I in presence of K_2CO_3 gave the fully protected ester **14** in moderate yield (51%) after preparative HPLC purification. In order to improve the yield, we have adopted a second approach: Boc-(*S*)-Arg(Pbf)-OH **10** was coupled under the same conditions as described above with fleshly prepared phosphono-aminoester TFA salt **12** using TBTU coupling agent**.** However, a mild increase in yield (3%) was observed.

Finally, deprotection of **14** to the target RZ-A **15** was performed in three steps: First, the two phosphonate methyl groups and Boc where removed using Me_3SiBr, followed by aqueous MeOH. According to MS (ESI^+) analysis, the Pbf moiety resisted to Me_3SiBr and was cleaved in a second step using $TFA/CH_2Cl_2/TIS/H_2O$:(19/19/1/1).[37] Finally, saponification of the methyl ester was performed using LiOH. The crude product was purified by semi-preparative C18-RP-HPLC and was lyophilized to give RZ-A **15** in moderate yield (47%). The obtained product was characterized with HRMS (ESI^+), 1D NMR (^1H, ^{31}P and ^{13}C, APT) and 2D NMR (^1H–^1H COSY, ^1H–^{13}C HSQC). These NMR data confirmed that we



Scheme 2. Synthesis of dipeptide RZ-A = (*S*)-Arg-(*S*,*Z*)-APPA **15**. Reagents and conditions: (a) TFA/CH_2Cl_2:(50/50); (b) TBTU or DEPBT, $KHCO_3$, THF/DMF:(4/1); (c) (i) H_5IO_6/CrO_3, CH_3CN/H_2O:(99/1), 0 °C; (ii) CH_3I, K_2CO_3, CH_3CN; (d) (i) 10% $(CH_3)_3SiBr$ in CH_2Cl_2 and then $MeOH/H_2O$:(9/1); (ii) $TFA/CH_2Cl_2/TIS/H_2O$:(19/19/1/1); (iii) 1 N LiOH; THF/H_2O:(4/1).

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have successfully synthesized the desired RZ-A **15** (Supplementary data). However, we have not found in the literature complete NMR spectra of RZ-A for comparison.

2.3. Synthesis of Plumbemycin A: PB-A 21

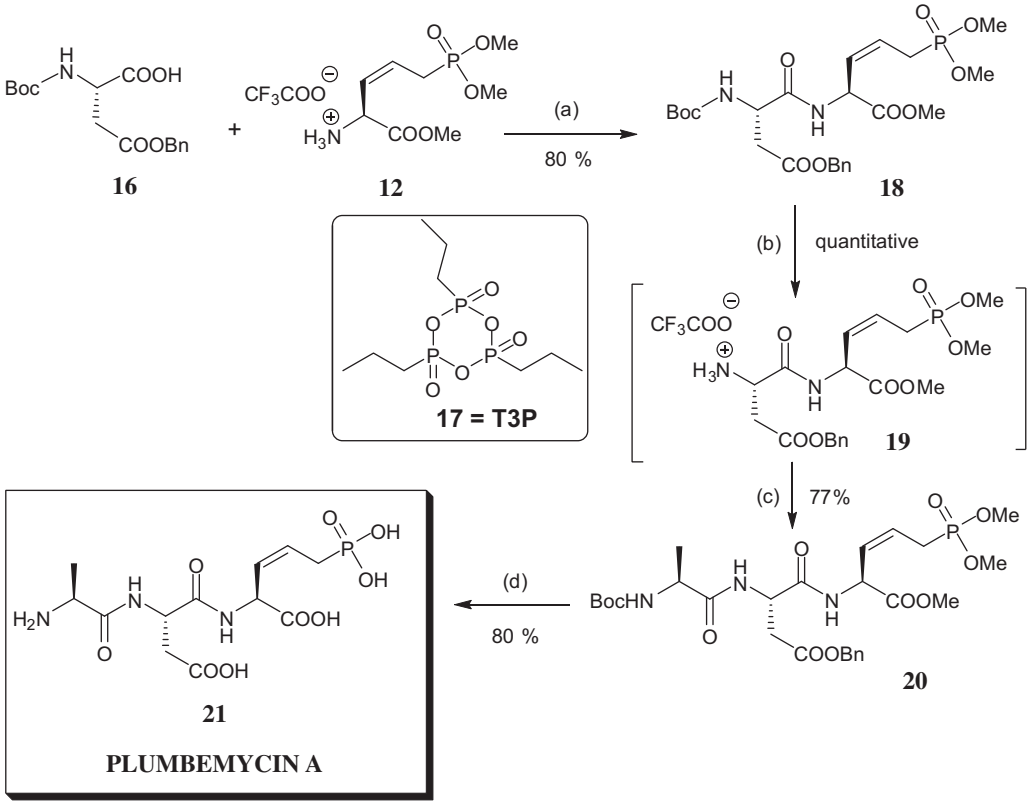
The same methodology used for the synthesis of RZ-A **15** was applied to prepare another (*S*,*Z*)-APPA containing molecule; the PB-A = (*S*)-Ala-(*S*)-Asp-(*S*,*Z*)-APPA **21**. The synthetic route to PB-A **21** is shown in Scheme 3. The first step of peptide coupling reaction was carried out using orthogonally protected Boc-(*S*)-Asp (Bn)-OH **16** and (*S*,*Z*)-APPA methyl ester **12** as starting materials. In this case, **T3P** (propanephosphonic anhydride)[38–41]**17** was used as coupling reagent in place of TBTU. In accordance to the literature, the use of T3P allowed an easier work-up and generally, higher yields were observed.

Thus, dipeptide **18** was synthesized by treating an equimolar mixture of the acid Boc-(*S*)-Asp(Bn)-OH **16** and **12** in DMF with 1.5 equiv of T3P **17** in presence of 2.5 equiv of *N*,*N*-diisopropylethylamine (DIEA) as base. After completion and standard work-up, flash chromatography was used to isolate dipeptide **18** in good yield (80%). The LRMS-(ESI$^+$) spectrum of **18** confirmed the expected mass. The Boc protective group of Asp amino acid in dipeptide **18** was then removed by TFA. The obtained crude salt **19** was used as obtained in a second peptide coupling reaction with Boc-(*S*)-Ala-OH under the same conditions as described above. After flash chromatography purification, the fully protected tripeptide **20** was obtained in 77% yield from **18**.

Finally, the target PB-A **21** = (*S*)-Ala-(*S*)-Asp-(*S*,*Z*)-APPA was obtained after *one pot* deprotection in two steps. First, the phosphonate methyl groups and the Boc were simultaneously removed by treating compound **20** with CH_3SiBr, followed by hydrolysis of the formed TMS ester with aq MeOH. Secondly, the benzyl and methyl esters functions of the crude corresponding phosphonic acid were saponified with LiOH. After purification by washing the aqueous solution three times with CH_2Cl_2, PB-A **21** was obtained as a white solid in good yield (80%) for the two steps of deprotection. The ^1H decoupled ^{31}P NMR of PB-A **21** showed one single peak at 22.39 ppm and the HRMS confirmed its expected mass. The ^1H NMR spectrum of PB-A **21** in D_2O (400 MHz) confirmed that the obtained product was the tripeptide Ala-Asp-(Z)-APPA. This spectrum was identical with that of PB-A purified from bacterial cultures and published by Park et al. in 1977.[13] Additionally, we have completed the characterization of the obtained PB-A **21** by ^{13}C and 2D NMR spectra (COSY ^1H–^1H and HSQC ^1H–^{13}C). The assignments confirmed the structure of PB-A **21** shown in Scheme 3 (see experimental section and Supplementary data).

3. Biological evaluation of synthetic RZ-A 15 and PB-A 21

RZs and PBs share a common mechanism of biological action. They use the oligopeptide transport system to enter inside the cell, where they are hydrolyzed by oligopeptidases into the active (*S*,*Z*)-APPA **8** moiety and Arg for RZ-A or Ala-Asp for PB-A. (*S*,*Z*)-APPA **8** is an inhibitor of threonine synthase[42] and subsequently interferes with threonine synthesis in sensitive yeasts and bacteria. In previous publications, antimicrobial activity was demonstrated for RZs and PBs purified in small quantities from *B. subtilis* and *S. plumbeus* culture supernatants, respectively.[13,43,44] Indeed, antimicrobial activities of PB-B with concentrations higher than 10 μg/mL against *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas ovalis* were described.[44] According to Park, PB-A has almost the same activity as PB-B.[13] Crude RZ-A was active against various fungal strains like *Candida albicans* and *Saccharomyces cerevisiae* (MIC:



Scheme 3. Synthesis of tripeptide PB-A = (*S*)-Ala-(*S*)-Asp-(*S*,*Z*)-APPA **21** Reagents and conditions: (a) T3P **17** 50% wt in DMF, DIEA, DMF; (b) TFA/CH_2Cl_2:(50/50); (c) Boc-(*S*)-Ala-OH, T3P **17** 50% wt in DMF, DIEA, DMF; (d) (i) 10% $(CH_3)_3SiBr$ in CH_2Cl_2 and then MeOH/H_2O:(9/1); (ii) 1 N LiOH, THF/H_2O:(4/1).

0.35 μg/mL).[43] In the present study, similar experiments were carried out with RZ-A **15** and PB-A **21** using experimental procedures validated previously with antifungal and antibacterial compounds (namely mycosubtilin and amylolysin).[45,46] However, no significant antimicrobial activities were observed against selected fungal and bacterial strains even with concentrations up to 1000 times of the MICs described in literature for PBs[13,44] and for RZs,[43] respectively.

Kugler et al. has shown that Thr like amino acids and some oligopeptides could have an antagonistic effect on the antibiotic activity of RZ-A.[43] However, this was demonstrated with relatively high amino acid concentration. Indeed, the biological effect of RZ-A has been observed by Kluger et al. on Malt Extract Medium that contains free Thr and Thr-containing peptides. Nevertheless, MICs of synthesized RZ-A **15** and PB-A **21** were determined as described in the literature (i.e., on Malt Extract Medium[43] and Stephenson–Wetham[44] medium for RZ-A and PB-A, respectively). In addition, MIC determinations were also performed in media that do not contain any amino acid (YNB and Stephenson–Wetham for RZs and PBs, respectively) to avoid neutralization of the biological effect by Thr or Thr-containing peptides, but no antimicrobial activities were observed.

4. Conclusion

In this work, we present an efficient synthetic route for the preparation of (*S*,*Z*)-APPA **8** and its derivatives phosphono-oligopeptides Rhizocticin A **15** and Plumbemycin A **21**. Unfortunately, the biological experiments done in this preliminary study show that these chemically synthesized compounds had no antimicrobial activity against selected fungi and bacteria. Despite these disappointing results, threonine synthase remains an interesting target for the development of new antibiotics. However, until now only racemic preparations of *Z*- and *E*-APPA were used in enzymatic studies with TS.[16,47] The synthetic scheme developed in this work can be used for the preparation of different enantiomers of *Z*- and *E*-APPA. A detailed kinetic study of these molecules with TS *in vitro* can help to clarify the absolute configuration of APPA in biological active compounds in the future. The active forms of APPA can then be introduced in various peptides using automated solid-phase peptide synthesis (SPPS) and resulting peptides can be used for biological experiments.

5. Experimental section

5.1. Chemistry

5.1.1. General information

All reactions were monitored by thin layer chromatography (TLC) and/or High Performance Liquid Chromatography (HPLC). The TLC were carried out on pre-coated TLC sheets POLYGRAM SIL G/UV_{254} from MACHEREY-NAGEL GmbH. Spots were visualized with UV light or by spraying a mixture of EtOH/H_2SO_4/*p*-anisaldehyde:(18/1/1) with 20 μL of acetic acid. The HPLC chain from WATERS was equipated with an UV-Detector (PDA Waters 996). HPLC analysis of synthesized compounds and their semi-preparative purifications were performed on XTerra RP 18 columns: (4.6 × 150 mm, 3.5 μm, Waters) and (7.8 × 300 mm, 7.0 μm, Waters), respectively. Preparative HPLC purifications was conducted on a column of type NOVASEP LC50.500.VE150 (Ø 50 mm) with Merck silica gel 60 (0.015–0.040 mm) under a pressure of 50 bars. Routine column chromatography was performed on Acros silica gel (0.060–0.200 mm). The enantiomeric excesses of non polar compounds were determined by HPLC on OD-H chiral column (Chiralpack, 4.6 × 250 mm, 5 μm, Daicel Chemical Industries LTD). For polar compounds, Astec Chirobiotic T chiral column (25 cm × 4.6 mm, 5 μm, Sigma–Aldrich/Supelco) was used. The 1D (^{1}H, ^{13}C, ^{31}P, DEPT or APT) and 2D (COSY, HSQC, HMBC) NMR spectra were recorded on Brucker 250 or 400 MHz apparatus. Chemical shifts are expressed in parts per million (ppm) and the peak shapes are denoted as follow: s = singlet, d = doublet, dd = doublet of doublet, t = triplet, q = quadruplet, m = multiplet, br = broad; Spectra were recorded as solution in various solvants ($CDCl_3$, D_2O, MeOD) and those were used as internal references.[48] The NMR spectra were analyzed using MestReNova software. High Resolution Mass Spectra (HRMS) were determined using an ESI-FT-ICR mass spectrometer (SolariX, Brucker) utilizing H_2O/CH_3CN:(50/50) as eluent. Low Resolution Mass Spectra (LRMS) were obtained on a Thermoquest Finnigan TSQ 7000 spectrometer, utilizing electron spray ionization (ESI) and H_2O/CH_3CN:(50/50) eluent system with 0.1% HCOOH added to this solvent. All starting materials and reagents were generally purchased from Aldrich, Acros, Epsilon, VWR or IRIS companies and used as such.

5.1.2. (*S*,*Z*)-*tert*-Butyl 4-(3-methoxy-3-oxoprop-1-enyl)-2,2-dimethyloxazolidine-3-carboxylate (*S*,*Z*)-2

To an oily suspension of NaH 60% (2.1 g, 52.5 mmol) in THF (90 mL), at 0 °C, under nitrogen was added, dropwise, a solution of $(CF_3CH_2O)_2P(O)CH_2CO_2Me$ (10 mL, 52.3 mmol) in THF (60 mL). The solution was stirred at 0 °C for 30 min and then cooled to −78 °C. To this solution was added 18-Crown-6 (69 g, 261 mmol) in THF (150 mL) and then (*R*)-Garner's aldehyde **1** (9.2 g, 40.2 mmol) in THF (20 mL). The reaction mixture was stirred at the same temperature for 2 h and the reaction quenched with aqueous saturated NH_4Cl solution. The reaction mixture was extracted with Et_2O, dried over $MgSO_4$, and concentrated *under vacuum*. The crude residue was purified by column chromatography (silica gel, hexanes/Et_2O:(1/4)) to give product (*S*,*Z*)-**2** as white solid. Yield (9.39 g, 82%). TLC (hexane/Et_2O:1/1): R_f = 0.71. HPLC (CH_3CN/H_2O:50/50, 0.7 mL/min): t_R = 9.5 min. ^{1}H NMR (250 MHz, $CDCl_3$) δ (ppm) 6.22 (m, 1H, C*H*=CH–COOMe), 5.78 (d, $^3J_{H-H}$ = 11.4 Hz, 1H, CH=C*H*–COOMe), 5.34 (s, 1H, C*H*-α), 4.23 (t, $^3J_{H-H}$ = 7.8 Hz, 1 H, $CH_{2a}O$), 3.71 (dd, $^3J_{H-H}$ = 9.2 and $^4J_{H-H}$ = 3.1 Hz, 1H, $CH_{2b}O$), 3.65 (s, 3H, $COOCH_3$), 1.57 (s, 3H, C-CH_3), 1.50–1.22 (m, 12H, C-CH_3 and [$C(CH_3)_3$] Boc). ^{13}C NMR (63 MHz, $CDCl_3$) δ (ppm) 166.25 (*C*OOMe), 152.26 (*C*O Boc), 151.86/151.24 (rotamers, *C*H=CH–COOMe), 119.67/119.08 (rotamers, CH=*C*H–COOMe), 94.42/93.89 (rotamers, *C*-$(CH_3)_2$), 80.54/79.92 (rotamers, ([*C*$(CH_3)_3$]Boc), 69.03/68.80 (rotamers, *C*H_2O), 56.61/55.58 (rotamers, *C*H-α), 51.38 ($COOCH_3$), 28.34 ([$C(CH_3)_3$] Boc), 27.32/26.62 (rotamers, C-CH_3), 24.92/23.75 (rotamers, C-CH_3). The enantiomeric excess of (*S*,*Z*)-**2** was determined by HPLC (Chiral OD-H column, *i*-PrOH/hexane:5/95), t_R = 7.8 min for (*R*) enantiomer and t_R = 8.5 min for (*S*) enantiomer in the HPLC chromatogram. The ee for (*S*,*Z*)-**2** was 94% and its $[\alpha]_D^{25}$ −27.3 (*c* = 0.91, $CHCl_3$). For (*R*,*Z*)-**2** enantiomer the ee = 96% and the measured $[\alpha]_D^{25}$ +28.5 (*c* = 1.03, $CHCl_3$). LRMS-(ESI$^+$), *m*/*z*: 286.2 $[M+H]^+$, 308.1 $[M+Na]^+$.

5.1.3. (*S*,*Z*)-*tert*-Butyl 4-(3-hydroxyprop-1-enyl)-2,2-dimethyloxazolidine-3-carboxylate 3

To a stirred solution of the ester (*S*,*Z*)-**2** (6.01 g, 21.1 mmol) in toluene (90 mL) at −78 °C under nitrogen was added a 1 M hexanes solution of DIBAL-H (63 mL, 63 mmol). The reaction mixture was stirred at −78 °C for 2 h and then quenched by dropwise addition of MeOH (14 mL). The mixture was warmed to room temperature; then ice (9 g) was added. The reaction mixture was stirred for 30 min. To the resulting white suspension was added $MgSO_4$ (23 g). After stirring for 30 min, the mixture was first filtered through a pad of $MgSO_4$ and then through silica gel to eliminate trace of aluminates. The filtrate was concentrated *under vacuum*

to give pure alcohol **7** as colorless oil. Yield (4.88 g, 90%). TLC (hexane/AcOEt: 80/20): R_f = 0.20. HPLC (CH_3CN/H_2O:50/50, 0.7 mL/min): t_R = 5.1 min. ^1H NMR (250 MHz, $CDCl_3$) δ (ppm) 5.81 (m, 1H, CH=C*H*–CH_2OH), 5.51 (t, $^3J_{H-H}$ = 10.6 Hz, 1H, C*H*=CH–CH_2-OH), 4.89 (m, 1H, C*H*-α), 4.41 (dd, $^3J_{H-H}$ = 12.7 and $^4J_{H-H}$ = 8.7 Hz, 1H, $CH_{2a}OH$), 4.03 (dd, $^3J_{H-H}$ = 9.0 and $^4J_{H-H}$ = 5.9 Hz, 1H, $CH_{2a}O$), 3.87 (dd, $^3J_{H-H}$ = 12.9 and $^4J_{H-H}$ = 5.7 Hz, 1H, $CH_{2b}OH$), 3.68 (dd, $^3J_{H-H}$ = 9.0 and $^4J_{H-H}$ = 1.8 Hz, 1H, $CH_{2b}O$), 1.54 (s, 3H, C-CH_3), 1.49–1.37 (m, 12H, C-CH_3 and [$C(CH_3)_3$]Boc). ^{13}C NMR (63 MHz, $CDCl_3$) δ (ppm) 152.36 (*C*O, Boc), 130.87 (CH=*C*H–CH_2OH), 130.23 (*C*H=CH–CH_2OH), 93.57 (*C*-$(CH_3)_2$), 81.12 ([*C*$(CH_3)_3$]Boc), 68.01 (CH_2O), 57.69 (*C*H_2OH), 53.64 (*C*H-α), 28.51 [$C(CH_3)_3$], Boc), 27.68 (C-CH_3), 24.84 (C-*C*H_3). LRMS-(ESI^+), *m*/*z*: 258.2 $[M+H]^+$, 280.0 $[M+Na]^+$.

5.1.4. (*S*,*Z*)-*tert*-Butyl 4-(3-bromoprop-1-enyl)-2,2-dimethyloxazolidine-3-carboxylate 4

The allyl alcohol **3** (4.4 g, 17 mmol) was dissolved in CH_2Cl_2 (30 mL) and then stirred under nitrogen at 0 °C. To this stirring solution were added solid CBr_4 (6.70 g, 21.3 mmol) followed by PPh_3 (7.10 g, 25.5 mmol). After stirring for 20 min, TLC analysis showed that the starting allyl alcohol **3** had completely reacted. The reaction mixture was added to a saturated solution of aq. $NaHCO_3$ and extracted with CH_2Cl_2. The combined organic layer was washed with brine, dried over $MgSO_4$, filtered and evaporated *under vacuum*. The residue containing allyl bromide and Ph_3P=O as by-product was purified by flash chromatography (silica gel, hexane/AcOEt: 70/30) to give pure bromide **4** as colorless oil. Yield (5.09 g, 93%). TLC (hexane/EtOAc: 80/20): R_f = 0.53. HPLC (CH_3CN/H_2O: 50/50, 0.7 mL/min): t_R = 13.3 min ^1H NMR (250 MHz, $CDCl_3$) δ (ppm) 5.80 (s, 1H, CH=C*H*–CH_2Br), 5.57 (t, $^3J_{H-H}$ = 10.2 Hz, 1H, C*H*=CH–CH_2Br), 4.69 (s, 1H-α), 4.38 (s, 1H, $CH_{2a}Br$), 4.11 (dd, $^3J_{H-H}$ = 8.9 and $^4J_{H-H}$ = 6.1 Hz, 1H, $CH_{2a}O$), 3.91 (s, 1H, $CH_{2b}Br$), 3.70 (dd, $^3J_{H-H}$ = 8.9 and $^4J_{H-H}$ = 2.6 Hz, 1H, $CH_{2b}O$), 1.60–1.39 (m, 15H, C-$(CH_3)_2$ and ([$C(CH_3)_3$], Boc).

5.1.5. (*S*,*Z*)-*tert*-Butyl 4-(3-(dimethoxyphosphoryl) prop-1-enyl)-2,2-dimethyloxazolidine-3-carboxylate 5

The obtained (*S*,*Z*)-allyl bromide **4** (4.90 g, 15.32 mmol) was mixed with an excess of $P(OMe)_3$ (5 mL) in a double necked 50 mL round bottomed flask attached to a condenser, with drying tube. The flask was heated at 100 °C and allowed to stir for 8 h. After this time, TLC analysis (hexane/EtOAc: 70/30) showed that the starting material **4** (R_f = 0.64) had completely reacted and a new compound formed (R_f = 0). A *vacuum* was used to remove the excess of $P(OMe)_3$ from the solution and the crude product was purified by flash column chromatography (silica gel, 30% EtOAc/Hexane and then 10% MeOH/EtOAc) to yield pure phosphonate **5** as a colorless oil. Yield (4.39 g, 82%). TLC (AcOEt/MeOH: 1/9), R_f = 0.54. HPLC (CH_3CN/H_2O:40/60, 0.7 mL/min): t_R = 7.5 min. ^1H NMR (400 MHz, $CDCl_3$) δ (ppm) 5.50 (m, 1H, CH=C*H*–CH_2P), 5.37 (s, 1H, C*H*=CH–CH_2P), 4.51 (s, 0.49H, C*H*-α), 4.41 (s, 0.47H, C*H*-α), 3.96 (m, 1H, $CH_{2a}O$), 3.63-3.55 (m, 7H, $CH_{2b}O$ and [P-$(OCH_3)_2$]), 3.02–2.23 (m, 2H, C*H*$_2$-P), 1.44 (m, 3H, C-*C*H_3), 1.36 (s, C-*C*H_3), 1.30 (m, 9H, [$C(CH_3)_3$]-Boc).^{13}C NMR (63 MHz, $CDCl_3$) δ (ppm) 151.87 (*C*O Boc), 134.98/133.71 (rotamers, CH=*C*H–CH_2P), 119.51/117.93 (rotamers, *C*H=CH–CH_2P), 94.17/93.47 (rotamers, *C*-$(CH_3)_2$), 79.95 ([*C*$(CH_3)_3$]Boc), 68.31 (rotamers, *C*H_2O), 54.20 (*C*H-α), 52.90/52.49 (2C, [P-$(OCH_3)_2$]), 28.45 (3C, [$C(CH_3)_3$], Boc), 27.42/26.49 (rotamers, C-*C*H_3), 24.79/23.59 (rotamers, C-*C*H_3), 24.71 (d, $^1J_{C-P}$ = 142 Hz, *C*H_2P). ^{31}P NMR (101 MHz, $CDCl_3$) δ (ppm) 30.08/29.29 (rotamers). LRMS-(ESI^+), *m*/*z*: 350.1 $[M+H]^+$, 372.0 $[M+Na]^+$. HRMS (ESI^+), *m*/*z* calculated for $C_{15}H_{29}NO_6P$ $[M+H]^+$ 350.1727. Found 350.1725.

5.1.6. (*S*,*Z*)-*tert*-Butyl 5-(dimethoxyphosphoryl)-1-hydroxypent-3-en-2-ylcarbamate 6

Oxazolidine phosphonate **5** (1.34 g, 3.84 mmol) was dissolved in 90% MeOH/H_2O (10 mL). To this solution was added Dowex 50XW H^+ resin (1.34 g) previously washed with MeOH and H_2O. The suspension was stirred over night at room temperature. After this time, TLC and HPLC analysis showed that the starting material had completely reacted. Filtration of the resin and removal of the solvent *under vacuum* provide a product which was purified using column chromatography (silica gel, $CHCl_3$/MeOH: 90/10) to afford pure **6** as a colorless oil. Yield (0.94 g, 79%). TLC ($CHCl_3$/MeOH: 9/1), R_f = 0.23. HPLC (CH_3CN/H_2O: 50/50, 0.7 mL/min): t_R = 3.6 min. ^1H NMR (400 MHz, $CDCl_3$) δ (ppm) 5.57–5.41 (m, 2H, C*H*=C*H*), 5.29 (d, $^3J_{N-H}$ = 7.4 Hz, 1H, N*H*), 4.31 (s, 1H, C*H*-α), 3.81 (s, 1H, CH_2OH), 3.66 (d, $^3J_{P-H}$ = 1.4 Hz, 3H, P-OCH_3), 3.63 (d, $^3J_{P-H}$ = 1.4 Hz, 3H, P-OCH_3), 3.52 (m, 2H, CH_2OH), 2.85 (m, 1H, CH_{2a}-P), 2.58 (m, 1H, CH_{2b}-P), 1.32 (s, 9H, [$C(CH_3)_3$], Boc). ^{13}C NMR (63 MHz, $CDCl_3$) δ (ppm) 155.80 (*C*O, Boc), 133.03 (d, $^2J_{C-P}$ = 14.4 Hz, CH=*C*H–CH_2P), 120.71 (d, $^3J_{C-P}$ = 10.7 Hz, *C*H=CH–CH_2P), 79.71 ([*C*$(CH_3)_3$], Boc) 65.00 (*C*H_2OH), 53.09 (d, $^3J_{C-P}$ = 6.9 Hz, P-O*C*H_3), 52.90 (d, $^3J_{C-P}$ = 6.8 Hz, P-O*C*H_3). 50.14 (s, *C*H-α), 28.46 ([$C(CH_3)_3$] Boc), 25.25 (d, $^1J_{C-P}$ = 139.3 Hz, *C*H_2-P). ^{31}P NMR (101 MHz, $CDCl_3$) δ (ppm) 30.14. $[\alpha]_D^{25}$ −91.7 (*c* = 0.88, $CHCl_3$). LRMS-(ESI^+), *m*/*z* calculated for $C_{12}H_{24}NO_6P$: 309.1; *m*/*z*: 309.9 $[M+H]^+$, 209.7 $[M-Boc+H]^+$.

5.1.7. (*S*,*Z*)-Methyl 2-(*tert*-butoxycarbonylamino)-5-(dimethoxyphosphoryl) pent-3-enoate 7

The oxidant reagent was prepared by dissolving H_5IO_6 (1.14 g, 5 mmol) and CrO_3 (2.3 mg, 0.12 mmol) in a volume of 99% aqueous CH_3CN (11.4 mL). Amino alcohol **6** (436 mg, 1.41 mmol) was dissolved in 99 % aq CH_3CN (4 mL), cooled to 0 °C and treated with the oxidant reagent (4 mL) in dropwise manner (30 min). After an additional stirring of 3 h at 0 °C, the mixture was added to a solution of saturated aq NaCl and extracted with ethyl acetate. The combined organic layer was dried over $MgSO_4$, filtered and evaporated *under vacuum* to give crude acid. This crude acid was dissolved in CH_3CN (5 mL), and to this solution were added K_2CO_3 (0.40 g, 2.89 mmol) and CH_3I (0.5 mL, 8 mmol) at room temperature. The mixture was left to react for 6 h and then solvents were evaporated *under vacuum*. The residue was dissolved in AcOEt and washed with an aqueous saturated solution of $NaHCO_3$ and then with brine. The organic layer was dried over $MgSO_4$, filtered and then evaporated. Subsequent purification by column chromatography (silica gel, $CHCl_3$/MeOH: 90/10) provided pure phosphonoester **7** as a colorless oil. Yield (228 mg, 50%). TLC ($CHCl_3$/MeOH: 9/1), R_f = 0.47. HPLC (CH_3CN/H_2O: 50/50, 0.7 mL/min): t_R = 5.3 min. ^1H NMR (400 MHz, $CDCl_3$) δ (ppm) 5.71 (m, 1H, CH=C*H*–CH_2P), 5.50 (m, 1H, C*H*=CH–CH_2P), 5.36 (d, $^3J_{N-H}$ = 7.4 Hz, 1H, N*H*)), 4.95 (t, $^3J_{H-H}$ = 7.3 Hz, 1H, C*H*-α), 3.74 (m, 3H, $COOCH_3$), 3.71 (s, 6H, P-$(OCH_3)_2$), 2.92–2.60 (m, 2H, C*H*$_2$-P), 1.40 (s, 9H, [$C(CH_3)_3$] Boc). ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm) 171.53 (*C*OOMe), 155.01 (*C*O, Boc), 128.38 (d, $^2J_{C-P}$ = 14.7 Hz, CH=*C*H–CH_2P), 124.47 (d, $^3J_{C-P}$ = 10.5 Hz, *C*H=CH–CH_2P), 80.18 ([*C*$(CH_3)_3$] Boc), 52.89 (P-OCH_3), 52.82 (P-OCH_3), 52.74 ($COOCH_3$), 51.43 (CH-α), 28.35 ([$C(CH_3)_3$], Boc), 25.45 (d, $^1J_{C-P}$ = 140.2 Hz, *C*H_2-P). $[\alpha]_D^{25}$ −57.8 (*c* = 1.00, $CHCl_3$). LRMS-(ESI^+); *m*/*z*: 337.8 $[M+H]^+$, 237.8 $[M+H-Boc]^+$, 697.2 $[2\,M+Na]^+$. ^{31}P NMR (101 MHz, $CDCl_3$) δ = 28.99 ppm.

5.1.8. (*S*,*Z*)-2-amino-5-phosphonopent-3-enoic acid: (*S*,*Z*)-APPA 8

Fully protected phosphono ester **7** (102 mg, 0.3 mmol) was dissolved in dried CH_2Cl_2 (1.8 mL) and TMSBr (0.2 mL) was added at 0 °C under nitrogen. The solution was allowed to stir overnight. The solvent and volatiles were evaporated under reduced pressure. The residue was dissolved in 90% aq MeOH and the solution

was allowed to stir for 1 h to ensure hydrolysis. The solvents were concentrated *under vacuum* and the residue co-evaporated with methanol and diethyl ether until a solid was obtained. This solid was dissolved in H_2O/THF:1/4 (2 mL) and then aq solution of LiOH (1 M, 0.5 mL) was added and the reaction mixture stirred at room temperature until the reaction was finished (HPLC monitoring, 3 h).The mixture was neutralized with Dowex, 50W × 4 H^+ resin, filtrated and evaporated to dryness. The residue was dissolved in water and pured in a funnel and washed with CH_2Cl_2 to yield the target compound (*S,Z*)-APPA **8** as a white solid after lyophilisation. Yield (47 mg, 80%). TLC (*n*-Butanol/pyridine/AcOH/H_2O:15/12/3/10), R_f = 0.24. HPLC (gradient 100% CH_3CN to 50 % H_2O (0.1% TFA), 30 min, flow: 0.7 mL/min): t_R = 12.4 min. ^1H NMR (400 MHz, D_2O): δ(ppm) = 6.03 (m, 1H, CH=C*H*–CH_2P), 5.56 (m, 1H, C*H*=CH–CH_2P) 4.65 (d, $^3J_{H-H}$ = 9.5 Hz, C*H*–CH=CH), 2.8–2.5 (m, 2H, CH_2P). ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm) 172.35 (d, $^5J_{C-P}$ = 1.5 Hz, *C*O), 131.79 (d, $^2J_{C-P}$ = 10.6 Hz, CH=*C*H–CH_2P), 122.82 (d, $^3J_{C-P}$ = 12.7 Hz, *C*H=CH–CH_2P), 50.71 (d, $^4J_{C-P}$ = 2.0 Hz, *C*H–CH=CH), 28.36 (d, $^1J_{C-P}$ = 127.0 Hz, *C*H_2P). ^{31}P NMR (101 MHz, D_2O): δ (ppm) = 19.20 (s). LRMS-(ESI^-), *m/z:* 194.1 $[M-H]^-$.

5.1.9. Fmoc-(*S,Z*)-APPA 9

(*S,Z*)-APPA **8** (23 mg, 0.12 mmol) and $NaHCO_3$ (40 mg, 0.49 mmol) were dissolved in 2 mL of water under stirring. A solution of Fmoc-OSu (41 mg, 0.12 mmol) dissolved in 2 mL of acetone was then added. After being stirred at room temperature overnight, the reaction mixture was concentrated. The crude product was dissolved with 1 N HCl (20 mL) and then extracted with AcOEt (3 × 20 mL). The organic layers were combined and evaporated *under vacuum* to give a crude product, which was purified by HPLC using semi-preparative XTerra column (eluent: CH_3CN/H_2O+0.1% TFA: 50/50, flow = 3 mL/min) to afford Fmoc-(*S,Z*)-APPA **9** as a white solid. Yield (24 mg, 69%). TLC ($CHCl_3$/MeOH: 9/1), R_f = 0.59. HPLC (CH_3CN/H_2O +0.1% TFA: 50/50, 0.7 mL/min): t_R = 7.6 min. ^1H NMR (250 MHz, MeOD) δ (ppm) 7.74 (d, $^3J_{H-H}$ = 7.3 Hz, 2H Ar Fmoc), 7.66–7.55 (m, 2H, Ar, Fmoc), 7.30 (m, 4H, Ar, Fmoc), 5.89–5.69 (m, 1H, CH=C*H*–CH_2P), 5.58 (m, 1H, C*H*=CH–CH_2P), 5.03 (d, $^3J_{H-H}$ = 9.5 Hz, 1H, CH-α), 4.31 (m, 2H, CH_2O Fmoc), 4.19 (m, 1H, C*H*–CH_2O, Fmoc), 2.85–2.55 (br m, 2H, CH_2-P). ^{31}P NMR (101 MHz, MeOD) δ 24.35 (s). $[\alpha]_D^{25}$ −15.9 (*c* = 0.46, AcOEt). LRMS-(ESI^+), *m/z*: 418.1 $[M+H]^+$. HRMS (ESI^-), *m/z* calculated for $C_{20}H_{21}NO_7P$ $[M-H]^-$, 416.0899. Found 416.0904. The enantiomeric excesses of Fmoc-(*S,Z*)-APPA were determined by HPLC (Chiral Astec Chirobiotic T column, MeOH/acetate buffer pH 4.5:20/80, flow = 1 mL/min). The ee of Fmoc-(*S,Z*)-APPA was 90% with t_R = 4.6 min for the major (*S*) enantiomer and t_R = 7.2 min for the (*R*) enantiomer.

5.1.10. Boc-(*S*)-Arg(Pbf)-(*S,Z*)-APPA-ol 13

Phosphonate oxazolidine **5** (1.75 g, 5 mmol) was deprotected with a 50% TFA solution in CH_2Cl_2 at room temperature. After 30 min, TLC analysis (AcOEt/MeOH: 9/1) indicated that the reaction was finished. The solvents were evaporated and toluene (10 mL) was added to form a TFA azeotrope and was removed *under vacuum*. The obtained crude TFA salt **11** was then dissolved in DMF (6 mL) and was added to a stirring solution of Boc-(*S*)-Arg(Pbf)-OH **10** (2.11 g, 4 mmol) in THF (24 mL). To this solution was added successively TBTU (1.29 g, 4 mmol), solid $KHCO_3$ (0.80 g, 8 mmol) and the reaction mixture was left to turn for 18 h at room temperature. After this time, the solvents were removed *under vacuum*. The crude product was dissolved with ethyl acetate (100 mL) and washed successively with saturated aqueous $NaHCO_3$, 0.5 N HCl and brine. The organic layer was dried over $MgSO_4$, filtered and evaporated *under vacuum* to give an off-white foam, which was further purified by preparative HPLC (silica gel, AcOEt/MeOH: 90/10) to provide the dipeptidol **13** as a white solid. Yield (1.87 g, 65%). TLC (AcOEt/MeOH: 1/9), R_f = 0.54. HPLC (CH_3CN/H_2O: 50/50, 0.7 mL/min): t_R = 5.0 min. ^1H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.43 (d, $^3J_{N-H}$ = 7.7 Hz, 1H, NH APPA ol), 6.37 (s, 3H, 3 × NH guanidine), 5.90 (d, $^3J_{N-H}$ = 6.4 Hz, 1H, NH Arg), 5.70–5.60 (m, 1H, CH=C*H*–CH_2P), 5.60–5.48 (m, 1H, C*H*=CH–CH_2P), 4.75 (s, 1H, C*H*-α APPA-ol), 4.22–4.03 (m, 2H, C*H*-α Arg and CH_2-O*H*), 3.74 (d, $^3J_{H-P}$ = 2.0 Hz, 3H, P-O-CH_3), 3.71 (d, $^3J_{H-P}$ = 1.9 Hz, 3H), P-O-CH_3), 3.66 (d, $^3J_{H-H}$ = 5.1 Hz, 2H, CH_2OH), 3.23 (s, 2H, CH_2-NH, Arg), 2.95 (s, 2H, CH_2, Pbf), 2.84–2.68 (m, 2H, CH_2-P), 2.57 (s, 3H, CH_3, Pbf)), 2.50 (s, 3H, CH_3, Pbf), 2.08 (s, 3H, CH_3, Pbf)), 1.80 (s, 1H, C$H_{2\beta}$ Arg), 1.67–1.53 (m, 3H, C$H_{2\beta}$ and C$H_{2\gamma}$-Arg), 1.45 (s, 6H, 2 × CH_3, Pbf), 1.39 (s, 9H, [C(CH_3)$_3$], Boc). ^{13}C NMR (63 MHz, $CDCl_3$) δ 172.79 (*C*O Arg), 158.74 (Ar *C*–O Pbf), 156.65 (*C*O Boc), 156.01 (C guanidine), 138.27 (Ar *C*-SO_2 Pbf), 132.52 (Ar–*C* Pbf), 132.17 (Ar–*C* Pbf), 131.72 (d, $^2J_{C-P}$ = 14.3 Hz, CH=*C*H–CH_2P), 124.63 (Ar–*C* Pbf), 120.97 (d, $^3J_{C-P}$ = 10.6 Hz, *C*H=C*H*–CH_2P), 117.52 (Ar–*C* Pbf), 86.41 (*C*-$(CH_3)_2$ Pbf), 79.74 ([*C*$(CH_3)_3$], Boc), 63.99 (*C*H_2OH), 54.37 (CH-α Arg), 52.95 [(2C, P-$(OCH_3)_2$], 49.16 (*C*H-α APPA), 43.19 (*C*H_2 Pbf), 40.10 (*C*H_2-NH Arg), 29.66 (*C*H_2-β Arg), 28.58 (C-$(CH_3)_2$ Pbf), 28.28 ([C(*C*H_3)$_3$] Boc), 25.68 (*C*H_2-γ Arg), 24.75 (d, $^1J_{C-P}$ = 138.6 Hz, *C*H_2-P), 19.37 (*C*H_3 Pbf)), 18.02 (*C*H_3 Pbf), 12.48 (*C*H_3 Pbf). ^{31}P NMR (101 MHz, $CDCl_3$) δ 30.17 (s). LRMS-(ESI^+), *m/z*: 718.3 $[M+H]^+$, 740.0 $[M+Na]^+$. HRMS (ESI^+), *m/z* calculated for $C_{31}H_{53}N_5O_{10}PS$ $[M+H]^+$, 718.3245. Found 718.3244.

5.1.11. Boc-(*S*)-Arg(Pbf)-(*S,Z*)-APPA-ester 14

5.1.11.1. Method A. Boc-(*S*)-Arg(Pbf)-(*S,Z*)-APPA-ol **13** (360 mg, 0.50 mmol) was dissolved in CH_3CN (3 mL) cooled to 0 °C and the H_5IO_6/CrO_3 oxidant (3 mL) was added dropwise (30 min). After an additional stirring of 3 h at 0 °C, the mixture was added to a saturated solution aq NaCl and extracted with ethyl acetate. The combined organic layers were evaporated *under vaccum* to give crude acid. This crude acid was dissolved in CH_3CN (5 mL) and to this solution were added K_2CO_3 (140 mg, 1.01 mmol) and CH_3I (0.5 mL) at room temperature. The mixture was left to react for 6 h and then solvents were evaporated. The residue was dissolved in ethyl acetate and washed with saturated aqueous $NaHCO_3$. The two phases were separated and the aqueous phase extracted with ethyl acetate two times. The combined organic layers were dried over $MgSO_4$, filtered and evaporated *under vaccum* to give a yellowish solid which was purified by preparative HPLC (silica gel, AcOEt/MeOH: 90/10) to provide compound **14** as a yellowish solid. Yield (192 mg, 51%).

5.1.11.2. Method B. Phosphono ester **7** (68 mg, 0.20 mmol) was deprotected with a 50% TFA solution in CH_2Cl_2. Boc-(*S*)-Arg(Pbf)-OH (105 mg, 0.2 mmol) and the obtained TFA salt methyl ester **12** were coupled by following the procedure 5.1.9. Yield (102 mg, 68%). TLC (CH_2Cl_2/MeOH: 1/9), R_f = 0.40. HPLC (CH_3CN/H_2O+0.1% TFA:40/60, 0.7 mL/min): t_R = 16.4 min ^1H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.74 (s, 1H, N*H* APPA), 6.32 (s, 3H, N*H* guanidine), 5.69 (s, 3H, C*H*=C*H* + N*H* Arg), 5.13 (s, 1H, C*H*-α APPA), 4.19 (m, 1H, C*H*-α Arg), 3.73 (s, 3H, COOCH_3), 3.70 (m, 6H, P-$(OCH_3)_2$), 3.21 (s, 2H, CH_2-NH Arg), 2.93 (s, 2H, CH_2 Pbf), 2.82 (m, 2H, CH_2-P), 2.55 (s, 3H, CH_3 Pbf), 2.48 (s, 3H, CH_3 Pbf), 2.06 (s, 3H, CH_3 Pbf), 1.79 (s, 1H, C$H_{2\text{-}\beta}$ Arg), 1.55 (m, 3H, C$H_{2\text{-}\beta}$ Arg and CH_2-γ Arg), 1.43 (s, 6H, C(CH_3)$_2$], Pbf), 1.38 (s, 9H, [C(CH_3)$_3$], Boc). ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm) 172.35 (*C*O), 170.84 (*C*O), 158.64 (Ar *C*–O Pbf)), 156.44 (*C*O Boc), 155.81 (*C* guanidine), 138.28 (Ar *C*-SO_2 Pbf), 132.99 (Ar-*C* Pbf), 132.22 (Ar–*C* Pbf), 127.89 (d, $^2J_{C-P}$ = 14.2 Hz, CH=CH–CH_2P), 124.53 (Ar–*C* Pbf), 124.28 (d, $^3J_{C-P}$ = 9.8 Hz, *C*H=CH–CH_2P), 117.40 (Ar–*C* Pbf), 86.33 (*C*$(CH_3)_2$ Pbf), 79.87 ([*C*$(CH_3)_3$], Boc), 68.13 (*C*H), 53.07 (d, $^3J_{H-P}$ = 1.4 Hz, P-O*C*H_3), 53.00 (d, $^3J_{H-P}$ = 1.4 Hz, P-O*C*H_3) 52.74 (O*C*H_3),

50.39 (*C*H-α APPA), 43.23 (*C*H_2-NH Arg), 29.66 (*C*H_2-β Arg), 28.58 ([C(*C*H_3)$_3$], Boc), 28.28 (C(*C*H_3)$_2$ Pbf), 25.81 (s), 25.24 (*C*H_2-γ Arg), 25.11 (d, $^1J_{C-P}$ = 140.39 Hz, *C*H_2P), 19.28 (*C*H_3 Pbf), 18.83 (*C*H_3), 17.92 (*C*H_3 Pbf), 12.45 (*C*H_3 Pbf). LRMS-(ESI$^+$); *m/z*: 746.4 [M+H]$^+$, 768.3 [M+Na]$^+$. HRMS (ESI$^+$), *m/z* calculated for $C_{32}H_{53}N_5O_{11}PS$ [M+H]$^+$, 746.3194. Found 746.3194.

5.1.12. RZ-A = (*S*)-Arg-(*S,Z*)-APPA 15

RZ-A **15** was obtained in three steps as follow: (1°) Boc-(*S*)-Arg(Pbf)-(*S,Z*)-APPA methyl ester dipeptide **14** (150 mg, 0.2 mmol) was dissolved in dried CH_2Cl_2 (1.8 mL) and the solution was cold down to 0 °C under nitrogen. Then, TMSBr (0.3 mL) was added and the solution was allowed to stir 6 h at room temperature. The volatiles were evaporated under reduced pressure and the residue was dissolved in 90% aq MeOH (3 mL). The solution was allowed to stir for 1 h to ensure hydrolysis and was concentrated. (2°) The crude product was dissolved by TFA/CH_2Cl_2/TIS/H_2O (9/9/1/1, 4 mL) stirred 2 h at room temperatureto remove the Pbf moiety and solvents were evaporated. (3°) The obtained crude product was redissolved in H_2O/THF:1/4 (2 mL) and then a solution of LiOH (1 M, 0.5 mL) was added and the reaction mixture was stirred at room temperature until the reaction was finished (HPLC monitoring). The mixture was neutralized with 0.2 N HCl, concentrated and purified on reverse phase semi preparative HPLC (C-18 Xterra column) using a gradient elution changed from 100% CH_3CN to 50% CH_3CN/H_2O +0.1% TFA: in 30 min, 3 mL/min yield **15** as a white solid after lyophilisation . Yield (33 mg, 47%). HPLC (gradient 100% CH_3CN to CH_3CN/H_2O +0.1% TFA: 50/50 for 30 min, 0.7 mL/min):t_R = 14.3 min. ^{1}H NMR + COSY (400 MHz, D_2O) δ (ppm) 5.86 (m, 1H, CH=C*H*–CH_2P), 5.64 (s, 1H, C*H*=CH–CH_2P), 5.14 (t, $^3J_{C-P}$ = 11.5 Hz, 1H, C*H*-α APPA), 3.98 (m, 1H, C*H*-α Arg), 3.16 (m, 2H, *C*H_2-δ Arg), 2.65 (dd, $^3J_{H-H}$ = 22.0 and $^4J_{H-H}$ = 8.0 Hz, 2H, C*H*$_2$-P), 1.92–1.79 (m, 2H, C*H*$_2$-β Arg), 1.75–1.49 (m, 2H, C*H*$_2$-γ Arg). ^{13}C NMR (63 MHz, D_2O) δ 173.67 (*C*OOH APPA), 168.95 (*C*ON Arg), 156.68 (*C* guanidine), 128.73 (d, $^2J_{C-P}$ = 10.7 Hz, CH=*C*H–CH_2P), 124.41 (d, $^3J_{C-P}$ = 13.5 Hz, *C*H=CH–CH_2P), 52.57 (*C*H-α APPA), 50.89 *C*H-α Arg), 40.31 (*C*H_2-δ Arg), 28.04 (d, $^1J_{C-P}$ = 130.41 Hz, *C*H_2P) 27.01 (*C*H_2-β Arg), 23.29 (*C*H_2-γ Arg). ^{31}P NMR (101 MHz, D_2O) δ (ppm) 21.98 (s). LRMS-(ESI$^+$), *m/z*: 352.3 (M+H)$^+$, HRMS (ESI$^+$), *m/z* calculated for $C_{11}H_{23}N_5O_6P$ [M+H] $^+$, 352.1380. Found, 352.1381

5.1.13. Tripeptide Boc-(*S*)-Ala-(*S*)-Asp (Bn)-(*S,Z*)-APPA methyl ester 20

A solution of Boc-(*S*)-Asp (Bn)-OH **16** (165 mg, 0.51 mmol) and the TFA salt of APPA methyl ester **12** (179 mg, 0.51 mmol) in DMF (10 mL) was cooled in an ice bath before addition of DIEA (0.175 mL, 2 mmol) under stirring. A solution of T3P **17** (50% wt in DMF, 0.45 mL, 0.77 mmol) was then added slowly. The mixture was removed from the cooling bath and allowed to stir overnight. The reaction was quenched with water (30 mL), and then the aqueous phase was extracted three by EtOAc (3 × 30 mL) and water (30 mL) and was stirred vigorously for 1 h. The two phases were separated and the aqueous phase extract twice with EtOAc (2 × 30 mL). The combined organic phases were washed with brine (30 mL) and concentrated to afford the crude peptide, which was purified by flash chromatography (silica gel, 100% EtOAc and then 10% MeOH in EtOAc) to yield dipeptide **18** as a yellowish solid. Yield (221 mg, 80%). The expected mass for this compound was confirmed by LRMS-(ESI$^+$), *m/z* calculated for $C_{24}H_{35}N_2O_{10}P$ [M+H]$^+$ 542.2; *m/z*: 543.1 [M+H]$^+$, 565.0 [M+Na]$^+$. The Boc protective group of the obtained dipeptide **18** (221 mg, 0.41 mmol) was deprotected with a 50% TFA solution in CH_2Cl_2 (6 mL) to provide the TFA salt **19**. This salt was dissolved in DMF (15 mL) upon addition of solid Boc-(*S*)-Ala-OH (77 mg, 0.41 mmol) and DIEA (0.14 mL, 0.82 mmol) under stirring. The solution was cooled down to 0 °C and then T3P 50% wt in DMF, 0.37 mL, 0.62 mmol) was added dropwise over 20 min. Then the reaction mixture was stirred for 24 h at room temperature. The reaction was quenched with water (20 mL) and then extracted with EtOAc (3 × 30 mL). The organic layer was washed with saturated $NaHCO_3$ (20 mL), 10% $NaHSO_4$ (20 mL), saturated NaCl (30 mL), dried over $MgSO_4$ and filtered before removing the solvent *under vacuum*. The crude product was purified by flash chromatography (silica gel, 100% EtOAc and then 10% MeOH/EtOAc) to yield the tripeptide **20** as a white solid. Yield (202 mg, 81%). TLC ($CHCl_3$/MeOH: 9/1), R_f = 0.47. HPLC (CH_3CN/H_2O: 40/60, 0.7 mL/min): t_R = 9.3 min. ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.69 (d, $^3J_{N-H}$ = 7.6 Hz, 1H, NH APPA), 7.55 (s, 1H, NH Asp), 7.41 (d, $^3J_{N-H}$ = 3.4 Hz, 1H, NH Ala), 7.27 (s, 5H, Ar Bn), 5.66 (s, 2H, C*H*=C*H*–CH_2P), 5.33 (s, 1H, N*H*), 5.05 (s, 3H, C*H*$_2$O and C*H*-α APPA), 4.84 (s, 1H, C*H*-α Asp), 4.10 (s, 1H, C*H*-α Ala), 3.68 (m, 9H, COOC*H*$_3$ and P-(OC*H*$_3$)$_2$), 3.00 (m, 1H, C*H*$_2$-β Asp), 2.74 (m, 3H, C*H*$_2$-β Asp and C*H*$_2$P), 1.37 (s, 9H, ([C(C*H*$_3$)$_3$], Boc) 1.30 (m, 3H, C*H*$_3$ Ala). ^{13}C NMR (63 MHz, $CDCl_3$) δ 172.91 (s), 171.30 (s), 170.57 (s), 169.99 (s), 155.54 (s), 135.44 (s), 128.50 (s), 128.25 (s), 128.21 (s), 128.10 (s), 127.87 (d, *J* = 14.9 Hz), 124.96 (d, *J* = 12.1 Hz), 80.05 (s), 66.62 (s), 52.87 (t, *J* = 6.7 Hz), 52.64 (s), 50.67 (s), 50.41 (d, *J* = 2.2 Hz), 49.10 (s), 35.74 (s), 28.27 (s), 25.48 (d, $^1J_{C-P}$ = 139.4 Hz), 18.24 (s), 18.24 (s), 15.22 (s). LRMS-(ESI$^+$), *m/z*: 514 [M-Boc+H]$^+$ 614.0 [M+H]$^+$, 636.0 [M+Na]$^+$ HRMS (ESI$^+$), *m/z* calculated for $C_{27}H_{41}N_3O_{11}P$ [M+H]$^+$, 614.2473. Found 614.2468.

5.1.14. PB-A = (*S*)-Ala-(*S*)-Asp-(*S,Z*)-APPA 21

Boc-(*S*)-Ala-(*S*)-Asp(Bn)-(*S,Z*)-APPA methyl ester **20** (123 mg, 0.2 mmol) was dissolved in dried CH_2Cl_2 (1.8 mL) and the solution was cold down to 0 °C under nitrogen. Then TMSBr (0.3 mL) was added and the solution was allowed to stir 6 h at room temperature. The volatiles were evaporated under reduced pressure and the residue was dissolved in 90% MeOH/H_2O (3 mL). The solution was allowed to stir for 1 h to ensure hydrolysis and 0.5 mL of a 1 N solution of LiOH in THF/H_2O:4/1 (0.5 mmol) was added and the reaction mixture stirred at room temperature until the reaction was finished (6 h). The mixture was acidified with 0.2 N HCl (20 mL), poured into a funnel and washed with CH_2Cl_2 (3 × 20 mL). The aqueous layer was concentrated under reduced pressure and PB-A **21** was obtained as a white solid after lyophilisation. Yield (61 mg, 80%). HPLC (CH_3CN/H_2O +0.1% TFA): 50/50, 0.7 mL/min): t_R = 2.3 min ^{1}H NMR (400 MHz, D_2O) δ (ppm) 5.98 (m, 1H, CH=C*H*–CH_2P), 5.76 (m, 1H, C*H*=CH–CH_2P), 5.24 (d, $^3J_{H-H}$ = 9.5 Hz, 1H, C*H*-α APPA), 4.91 (m, 1H, C*H*-α Asp), 4.26 (m, 1H, C*H*-α Ala), 3.06–2.93 (m, 2H, C*H*$_2$-β Asp), 2.86–2.66 (m, 2H, C*H*$_2$P), 1.63 (m, 3H, C*H*$_3$ Ala). ^{13}C NMR (63 MHz, $CDCl_3$) δ 174.15 (*C*OOH APPA), 173.58 (*C*OOH Asp), 171.46 (*C*ON Asp), 170.60 (*C*ON Ala), 128.04 (d, $^2J_{C-P}$ = 13.6 Hz, CH=CH–CH_2P), 125.18 (d, $^3J_{C-P}$ = 10.2 Hz, *C*H=CH–CH_2P), 50.99 (*C*H-α APPA), 49.60 (*C*H-α Asp), 49.23 (*C*H-α Ala), 36.42 (*C*H_2-β Asp), 28.01 (d, $^1J_{C-P}$ = 131.8 Hz, *C*H_2P), 16.79 (*C*H_3 Ala). ^{31}P NMR (101 MHz, D_2O) δ (ppm) 22.39 (s). LRMS-(ESI$^+$), *m/z*: 382.1 [M+H]$^+$. HRMS (ESI$^-$), *m/z* calculated for$C_{12}H_{19}N_3O_9P$ [M−H]$^-$, 380.0864. Found 380.0867.

5.2. Biology

5.2.1. Antibacterial and antifungal tests in liquid media

Antifungal activity of RZ-A **15** was tested against *Candida albicans*, *Saccharomyces cerevisiae* and *Candida glabrata* strains by a microdilution method as previously described[45,49] Briefly, indicator strains were aerobically grown in 24-wells culture plates in the presence of purified RZ-A **15** at concentrations ranging from 3.5 μg/L to 1050 μg/L in the appropriate culture medium. Each well was seeded at an optical density at 600 nm (OD_{600}) of 0.1. After

24 h of incubation under agitation at 28 °C, cell growth was estimated by mean of OD_{600} measurements. Each experiment was performed in triplicate in two complex medium namely: YPD (10 g/L yeast extract, 20 g/L casein peptone, 20% dextrose) and malt extract medium (20 g/L malt extract) as well as in two defined medium: RPMI 1640 (Sigma–Aldrich) and YNB without amino acids (Difco).

Similarly, antibacterial activity of PB-A **21** was tested against Gram-positive (*Bacillus subtilis*, *Listeria innocua*) and Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*). Cells were grown in the presence of purified PB-A **21** at concentration ranging from 2 μg/L to 10 mg/L in the appropriate culture medium at 37 °C for 24 h. Each experiment was performed in triplicate in two different medium: LB (casein tryptone 10 g/L, yeast extract 5 g/L and NaCl 10 g/L, agar 20 g/L) and Stephenson-Wetham medium (KH_2PO_4 1 g/L, $MgSO_4$ $7H_2O$ 0.7 g/L, NaCl 1 g/L, $(NH_4)_2HPO_4$ 4 g/L, $FeSO_4$ $7H_2O$ 0.03 g/L, glucose 5 g/L).

5.2.2. Antibacterial and antifungal tests on solid media

Antifungal and antimicrobial activity tests on plates were performed as previously described by Halimi et al in 2010.[46] Briefly, sterile paper disks (6 mm diameter) containing different amounts of RZ-A **15** or PB-A **21** were deposited on the surface of agar plates containing culture medium previously seeded with the indicator strains listed above. Antifungal and antibacterial activities were estimated by measuring the diameter of the growth inhibition zone obtained after 24 h of growth at 28 °C and 37 °C, respectively. The same media (supplemented with 20 g/L agar), indicator strains and concentrations of RZ-A **15** and PB-A **21** as those used for the micro dilution method were used in agar diffusion tests.

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Supplementary data

Supplementary data (1D NMR spectra: ^{1}H, ^{31}P, ^{13}C, DEPT or APT and 2D NMR spectra: ^{1}H–^{1}H COSY, ^{1}H–^{13}C HSQC of the key intermediates and of all news compounds) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.06.064.

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