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Synthesis and enzymatic evaluation of phosphoramidon and its β anomer: Anomerization of α-L-rhamnose triacetate upon phosphitylation

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ABSTRACT

A novel and efficient strategy for the synthesis of phosphoramidon and its β anomer has been developed by manipulating the anomerization of α-L-rhamnose triacetate upon phosphitylation. The experimental results suggest that proton transfer, bond rotation, and N atom are the key factors for the anomerization. The determined K_i and K_d values establish that phosphoramidon prepared by this method possesses excellent biological activity, and indicate that the contacts of rhamnose moiety with the enzyme have limited contribution to the binding.

1. Introduction

Phosphoramidon (**1**), a naturally occurring glycopeptide first isolated from a strain of *Streptomyces tanashiensis* by Umezawa and co-workers in 1972, has a unique chemical structure featuring a phosphoramidate linkage between α-L-rhamnose and L-leucine-L-tryptophan. As a microbial metabolite, phosphoramidon exhibits potent inhibitory activity against thermolysin, a zinc endopeptidase isolated from *Bacillus thermoproteolyticus* (K_i = 32 nM).[1] It is also identified as an inhibitor of endothelin converting enzyme (ECE) (IC_{50} = 0.69 μM), a zinc-containing enzyme responsible for activation of endothelin (ET-1). As a potent vasoconstrictor, ET-1 causes a variety of pathophysiological conditions, such as hypertension, stroke, and cardiac failure.[2] Phosphoramidon has been extensively utilized as a reference inhibitor for investigation of ECE-related diseases and development of novel therapeutic agents with better metabolic stability and ECE specificity.[3] Meanwhile, phosphoramidon inhibits other metalloproteases, such as neutral endopeptidase (NEP)[4] and bacterial elastases,[5] rendering this natural compound a valuable tool in biological and medicinal research.

Despite its versatile biological activities, only a few methods have been reported for the chemical synthesis of phosphoramidon and its analogues. These approaches involved different P(III) and P(V) intermediates, such as L-rhamnose-1-phosphate,[1b] dichlorophosphate,[6] *H*-phosphonate monoester,[7] and di(*p*-methoxy-benzyl)-*N*,*N*-diisopropylphosphoramidite.[8] However, all these methods afforded their target molecules in only low to moderate yields with complicated purification procedures. We report herein a novel and efficient route for the total synthesis of phosphoramidon and its β anomer by manipulating the anomerization of α-L-rhamnose triacetate upon phosphitylation.

2. Results and discussion

2.1. Synthesis of phosphoramidon and its β anomer

To overcome the problems of known methods, our synthetic strategy employs three building blocks, including α-L-rhamnose triacetate (**2**), benzyl-*N*,*N*-diisopropylchlorophosphoramidite (**3**), and NH_2-L-leucine-L-tryptophan benzyl ester (**4**), all of which can be efficiently prepared from inexpensive starting materials and contain easily removable protecting groups. As shown in Scheme 1, peracetylation of L-rhamnose (**5**) followed by selective deprotection of anomeric acetyl group afforded **2** as pure α anomer. Starting from PCl_3 (**7**), phosphitylating reagent **3** was obtained in high yield and purity based on a modified procedure in our work.[9] EDC coupling of *N*-Boc-L-leucine (**9**) and L-tryptophan-OBn (**10**) gave dipeptide **11**. Removal of Boc with TFA and simple alkaline workup afforded **4** in almost quantitative yield.

The construction of the phosphoramidate linkage was achieved efficiently via three consecutive steps, including phosphitylation of

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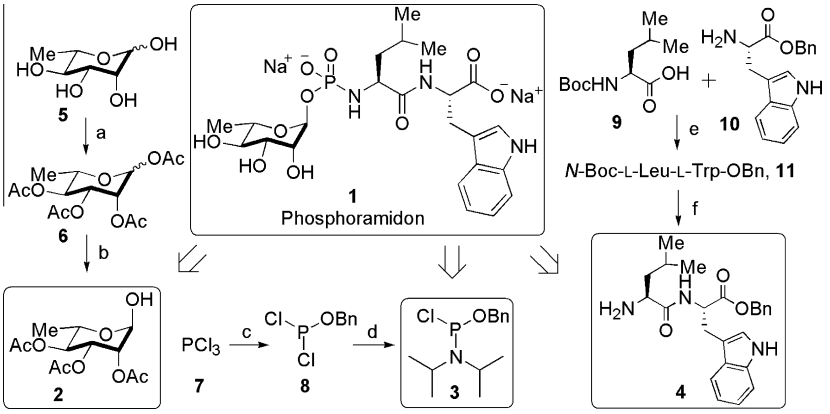
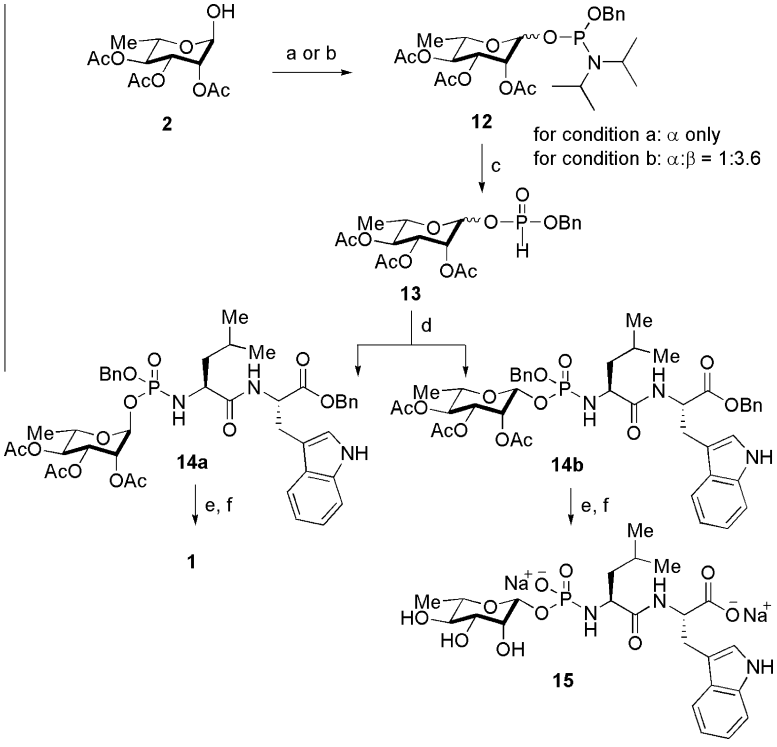


Scheme 1. Design and synthesis of the building blocks for phosphoramidon (**1**). Reagents and conditions: (a) Ac_2O, NaOAc, reflux, 4 h; (b) 1,2-diamino ethane, HOAc, THF, rt, 24 h (76% over 2 steps); (c) BnOH, CH_2Cl_2, 0 °C to rt, 6 h; (d) $(iPr)_2NH$, Et_3N, Et_2O, 0 °C to rt, 12 h (82% over 2 steps); (e) EDC, HOBt, CH_2Cl_2, rt, 12 h (89%); (f) 20% TFA, CH_2Cl_2, rt, 2 h (99%).

the anomeric OH of **2** with **3**, hydrolysis of phosphoramidite intermediate **12** with an acidic catalyst, and oxidative coupling of the resulting *H*-phosphonate diester **13** with **4** (Scheme 2). However, it was found that if the reaction was performed with DBU as base and diethyl ether as solvent at 0 °C in low concentration (condition a), α anomer of **12** was the sole product. In contrast, if the reaction was conducted with Et_3N as base and CH_2Cl_2 as solvent at 35 °C in high concentration (condition b), the β anomer in turn became the major product (α/β = 1:3.6).[10] After salt and extra base were removed, 1*H*-tetrazole-catalyzed hydrolysis of **12** prepared under either condition a or b afforded the corresponding batch of *H*-phosphonate diester **13**. Oxidative coupling of **13** with dipeptide **4** using CCl_4 and Et_3N gave phosphoramidate **14**. For the reaction based on condition a, **14a** was isolated in 72% yield (α only). Otherwise, a mixture of α and β anomers (**14a** and **14b**) was obtained, if **2** was phosphitylated under condition b. The α/β mixture was readily separable on silica gel chromatography, which afforded **14a** and **14b** in 16% and 53% yield (α/β = 1:3.3), respectively.

Final deprotection of **14a** and **14b** was conducted by catalytic hydrogenation and deacetylation with NaOMe in a one pot manner (Scheme 2). Due to the fact that phosphoramidate is susceptible to solvolysis at both low and high pH, two benzyl esters were rationally introduced in our design and avoided harsh deprotection conditions used in previous methods.[6,7b,11] Hydrogenation of **14a** and **14b** with 5% Pd/C in anhydrous MeOH removed the benzyl groups in 3 h. Then, a solution of NaOMe in MeOH was added in drops to reach a final concentration of 0.05 M and pH 9.0. The deacetylation completed in 3 h with only trace amounts of byprod-



Scheme 2. Synthesis of phosphoramidon (**1**) and its β anomer (**15**). Reagents and conditions: (a) **3**, DBU, Et_2O, 0 °C, 30 min, [**2**] = 0.057 M; (b) **3**, Et_3N, CH_2Cl_2, 35 °C, 30 min, [**2**] = 0.46 M; (c) 1*H*-tetrazole, H_2O, CH_3CN, rt, 30 min; (d) **4**, CCl_4, Et_3N, CH_3CN, 0 °C to rt, 30 min [72% for **14a** (α only, condition a)/16% for **14a** and 53% for **14b** (α/β = 1:3.3, condition b) over 3 steps]; (e) H_2, 5% Pd/C, MeOH, rt, 3 h; (f) NaOMe, MeOH, rt, 3 h (92% for **1**/93% for **15** over 2 steps).

ucts. It was found that when the final concentration of NaOMe exceeded 0.1 M, more byproducts appeared on TLC. After neutralization with diluted HCl in MeOH, crude **1** and **15** were easily purified by size-exclusion gel chromatography and recrystallization respectively in high yields. The purity of **1** and **15** determined by NMR and reverse-phase HPLC analysis was over 98%.

2.2. Mechanistic study and control of the anomerization of α-L-rhamnose triacetate upon phosphitylation

As mentioned above, the experimental results demonstrated the efficacy of combining phosphoramidite chemistry[12] and Atherton-Todd reaction[13] for the construction of phosphoramidate at the anomeric position of glycosyl substrates. However, to our surprise, when **2** (pure α anomer) was used in our initial attempt (Et_3N, CH_2Cl_2, 20 °C, [**2**] = 0.057 M, condition c in Table 1), **14a** and **14b** were isolated in nearly equal amount, which was an interesting phenomenon that has never been mentioned in literature.[9,14] ^{13}C NMR spectra of crude **12** and **13** were obtained, and the data showed that the α/β ratios were almost the same as **14a**/**14b**. Furthermore, no β anomer was observed on ^{13}C NMR spectrum, when **2** was treated with 1.0 equiv of HCl, Et_3N, or $Et_3N{\cdot}HCl$ respectively in CH_2Cl_2 after 12 h. These results suggested that the anomerization happened during the process of phosphitylation.

As illustrated in Scheme 3, we postulated that upon the substitution of chloride with anomeric OH group, if the resulting acidic proton was neutralized by the base, the α anomeric configuration remained untouched (**12a**, path A). However, the acidic proton may also be quickly transferred to the adjacent oxygen atom on C5, concerted with attack of nitrogen atom on phosphoramidite at C1, to cause pyranose ring opening (**17**). Rotation of C1–C2 bond (**18**) followed by attack of OH on C1 and reclosure of the ring inverted the anomeric configuration (**12b**, path B). Although the bond rotation may involve multiple factors, the steric repulsion between the *N*-isopropyl groups and acetyl group on C2 could be a possible driving force.

Table 1
Effects of solvent and base on anomerization of **12**[a]

Entry	Solvent	α/β ratio (Et_3N as base)	α/β ratio (DBU as base)
1	CH_2Cl_2	1.1:1 (condition c)	1.5:1
2	CH_3CN	1.3:1	2.1:1
3	THF	N.R.	3.0:1
4	Et_2O	N.R.	4.1:1

[a] All the reactions listed in Table 1 were performed at 20 °C with [**2**] = 0.057 M.

Table 2
Effect of temperature on anomerization of **12**[a]

Entry	Solvent/ Base	α/β ratio (20 °C)	α/β ratio (0 °C)	α/β ratio (−20 °C)
1	Et_2O/DBU	4.1:1	α only	—
2	THF/DBU	3.0:1	4.3:1	α only

[a] All the reactions listed in Table 2 were performed with [**2**] = 0.057 M.

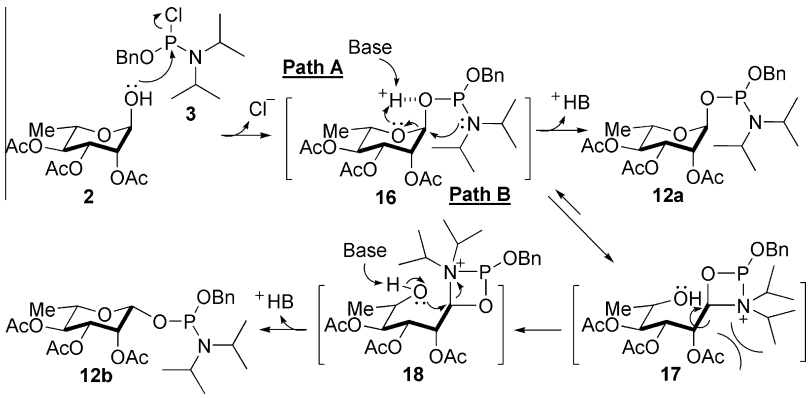
To validate the proposed mechanism, more experiments concerning the key factors, the acidic proton and bond rotation, were conducted. Based on the hypothesis that more basic reaction condition should reduce the proton transfer and formation of β anomer, the reaction was tested in different solvents with Et_3N or DBU as base. As expected (Table 1), the stronger basicity of DBU significantly increased the ratio of α anomer. Meanwhile, solvents that could serve as Lewis bases, such as THF and Et_2O, also lowered the portion of β anomer by quenching the acidic proton. The possibility of using weak organic bases, such as pyridine, lutidine, and *N*-methylimidazole, to favor the formation of **12b** in CH_2Cl_2 was also explored. But the reaction was sluggish in the absence of a strong base.

In the following research, the effect of reaction temperature on the α/β ratio of **12** was investigated. The reaction was performed at lower temperatures to slow down the rotation of C1–C2 bond, thereby decreasing the portion of β anomer. The data in Table 2 showed that when the reaction with Et_2O as solvent and DBU as base was lowered to 0 °C, α anomer of **12** was obtained as the only product (Scheme 2, condition a). The reaction with THF/DBU exhibited a similar trend, but required −20 °C to completely eliminate the generation of β anomer.

In contrast, the reaction of **2** and **3** in CH_2Cl_2 with Et_3N as base was performed at 35 °C to promote the formation of β anomer. Meanwhile, it was found that the concentration of **2** also played a key role in the anomerization. The data in Table 3 showed that both higher temperature and concentration favored the formation of β anomer. The lowest α/β ratio (1:3.6) was obtained, when the reaction was conducted at 35 °C with [**2**] = 0.46 M (Scheme 2, condition b).

2.3. Assignment of the anomeric configurations

In analysis of ^1H NMR data of **1** and **15**, we found that the $^3J_{H1,H2}$ and $^3J_{H1,P}$ values of **1** (0–1 and 7.6 Hz), which were used for assignment of the α anomeric configuration of **1** in previous reports,[1a,6–8] were only of slight difference to those of **15** (0–1 and 8.4 Hz) due to the axial OH at C2 position ($\Phi_{H1,H2}$ = 60°) of both **1** and **15**. Accord-



Scheme 3. A proposed mechanism for the anomerization of α-L-rhamnosyl moiety upon phosphitylation.

Table 3
Effects of temperature and concentration of **2** on anomerization of **12**[a]

Entry	[**2**] (M)	α/β ratio (20 °C)	α/β ratio (35 °C)
1	0.057	1:0.9	1:1.0
2	0.23	1:1.4	1:2.1
3	0.46[b]	—	1:3.6

[a] All the reactions listed in Table 3 were performed with CH_2Cl_2/Et_3N.
[b] The maximum concentration for phosphitylation reaction of **2** in CH_2Cl_2 at 35 °C.

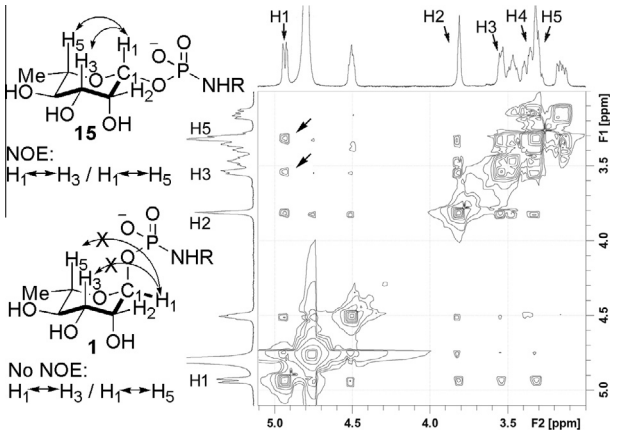


Figure 1. 2D NOSEY NMR spectrum of **15**.

ing to literature precedents, measuring one-bond C–H coupling constants is a reliable method to distinguish anomeric pairs in pyranoses.[15] The $^1J_{C1,H1}$ values of these two compounds (170 Hz for **1** and 159 Hz for **15**) matched well with the typical values of α- and β-glycosyl anomers (170 Hz for α and 160 Hz for β). In addition, two-dimensional NMR experiments unambiguously confirmed the β configuration of **15** by detection of a nuclear Overhauser effect (NOE) between H1 and H3/H5 (Fig. 1).

2.4. Synthesis of α- and β-L-rhamnosyl-1-phosphoramidates with control of anomeric configuration

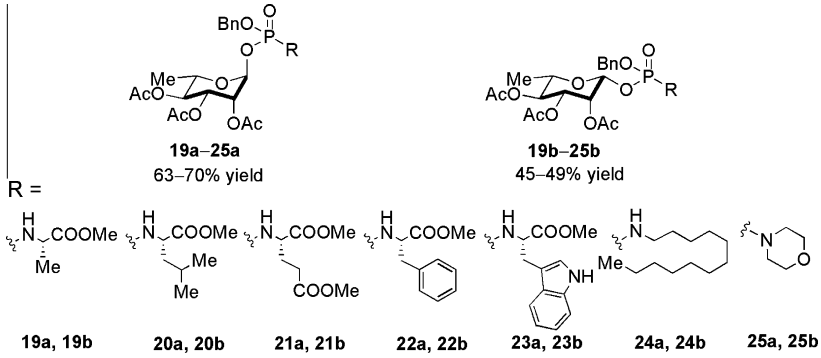
Our finding that anomerization of **2** upon reacting with chlorophosphoramidite **3** could be manipulated by using different reaction conditions provided a novel approach for constructing α and β phosphoramidate linkage at the anomeric position of rhamnose with control of stereochemistry. To prove this point, seven α-2,3,4-*O*-triacetyl-L-rhamnosyl-1-phosphoramidates (**19a**–**25a**) and their β anomers (**19b**–**25b**) were prepared according to this new method in good yields. Moreover, the $^1J_{C1,H1}$ values of these pairs provided valuable references for determination of the anomeric configurations of L-rhamnosyl-1-phosphoramidate derivatives (Table 4).

2.5. Enzymatic evaluation of phosphoramidon and its β anomer

To examine the biological activity of **1** and its β anomer **15** prepared by our new method, the inhibitory effects of these two compounds on thermolysin were investigated according to literature methods. The inhibitor constants (K_i) of **1** and **15** at pH 7.5 were determined based on the Henderson plots of inhibition of a substrate cleavage (Fig. 2).[1b,1c,16] The dissociation constants of the EI complexes (K_d) were calculated from the fluorometric titration curves by non-linear least-square method (Fig. 3).[17]

The K_i and K_d values of **1** obtained in our experiments were $(3.0 \pm 0.1) \times 10^{-8}$ M and $(3.2 \pm 0.6) \times 10^{-8}$ M respectively, and compared well with those reported previously.[1c,1d] It was determined that β anomer **15** also exhibited potent inhibitory activity against thermolysin with a K_i of $(2.8 \pm 0.8) \times 10^{-7}$ M and K_d of $(2.1 \pm 0.5) \times 10^{-7}$ M, which were about one order of magnitude higher than those of **1**, indicating that altering the α configuration of **1** to β only moderately decreased the binding affinity of **15** to thermolysin. This result confirmed that *N*-phosphoryl dipeptide moiety in **1** and **15** is the primary structural feature for effective inhibition, and the interactions between rhamnose moiety and S_1 subsite of thermolysin make only a relatively small contribution to the binding.[1c,1e,18]

Table 4
$^1J_{C1,H1}$ Values of **14a**,**b**, **19a**,**b**–**25a**,**b**



Compounds	$^1J_{C1,H1}$ of α anomer (Hz)	$^1J_{C1,H1}$ of β anomer (Hz)
14a/14b	176	162
19a/19b	176	164
20a/20b	171	161
21a/21b	176	163
22a/22b	176	161
23a/23b	177	161
24a/24b	175	162
25a/25b	175	161

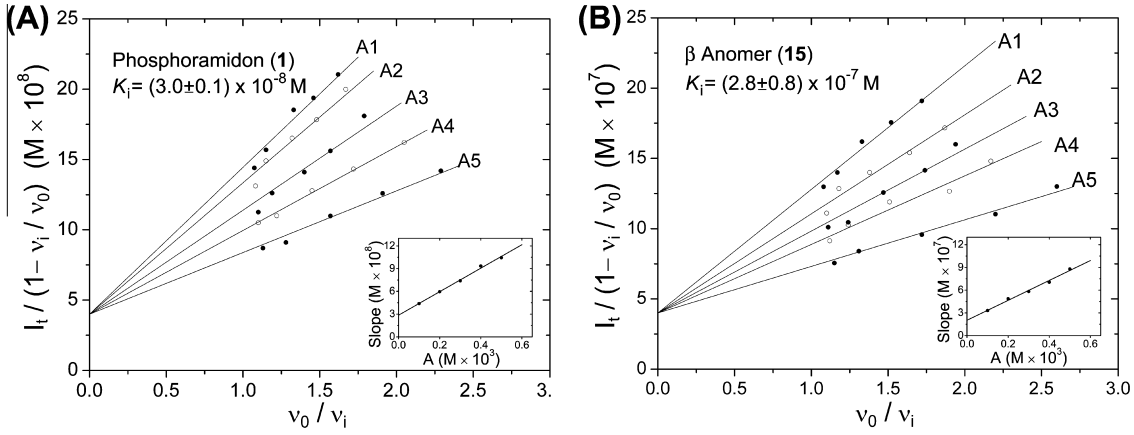


Figure 2. Henderson plots of **1** (A) and **15** (B) inhibition towards the cleavage of 3-(2-furylacryloyl)-glycyl-L-leucine amide (FAGLA) by thermolysin in pH 7.5 Tris buffer at 25 °C. $[E]_0 = 4.0 \times 10^{-8}$ M, $[A]_1 = 5.0 \times 10^{-4}$ M, $[A]_2 = 4.0 \times 10^{-4}$ M, $[A]_3 = 3.0 \times 10^{-4}$ M, $[A]_4 = 2.0 \times 10^{-4}$ M, $[A]_5 = 1.0 \times 10^{-4}$ M, $[I]_1 = 1.0 \times 10^{-8}$ M, $[I]_2 = 2.0 \times 10^{-8}$ M, $[I]_3 = 4.0 \times 10^{-8}$ M, $[I]_4 = 6.0 \times 10^{-8}$ M, $[I]_5 = 8.0 \times 10^{-8}$ M. I_t Indicates total concentration of inhibitor. υ_i and υ_0 Indicate velocity in the presence and in the absence of the inhibitor, respectively. The increasing slopes with increasing concentrations of A indicate that the inhibition is in a competitive manner.

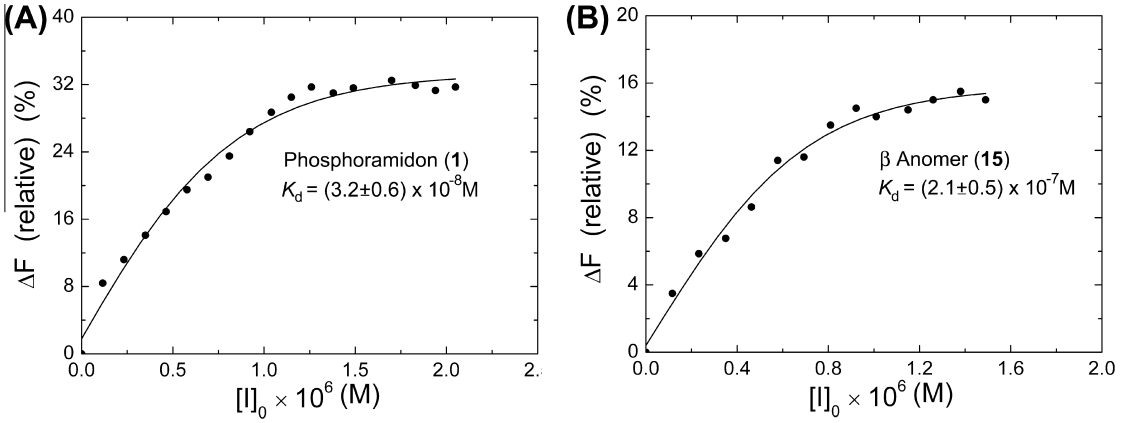


Figure 3. Fluorometric titration curves of [thermolysin-**1**] (A) and [thermolysin-**15**] (B) complexs in pH 7.5 Tris buffer at 25 °C. ΔF (relative) represents the relative increment of the fluorescence against the total fluorescence intensity of the enzyme and inhibitor at a concentration equal to that of the enzyme, ΔF (relative) = $\Delta F/(F_E + F_{I,eq})$. $[E]_0 = 1.15 \times 10^{-6}$ M, λ_{ex} = 280 nM, λ_{em} = 360 nM. K_d values were calculated by the non-linear least-squares method.

3. Conclusions

In summary, we developed a novel and efficient route for the total synthesis of phosphoramidon (**1**) and its β anomer (**15**). Comparing to the few known methods, this new route features easily accessible building blocks, efficient installation of phosphoramidate at anomeric position of α-L-rhamnose triacetate (**2**) with control of stereochemistry, fast removal of all protecting groups under mild conditions, and simple purification procedures. The mechanism of anomerization of **2** in the reaction with chlorophosphoramidite **3** was proposed to involve two steps, including the acidic proton transfer induced N atom attack at C1 and pyranose ring opening, and subsequent bond rotation and reclosure of the ring. The definite assignment of the anomeric configurations of **1** and **15** was achieved by measuring $^1J_{C1,H1}$ and NOE effect of H1 with H3/H5. Our finding that anomerization of α-L-rhamnose triacetate upon phosphitylation could be manipulated by using different reaction conditions offered a novel and efficient approach for constructing α- and β-glycosyl-1-phosphoramidate with control of anomeric configuration. The $^1J_{C1,H1}$ values of a series of novel L-rhamnosyl-1-phosphoramidates (**19a**,**b**–**25a**,**b**) prepared by this new method provide useful information for identification of the anomeric configurations of related phosphorus-containing glycosides. The K_i and K_d values of **1** and **15** determined by enzymatic assays reveal that the samples synthesized by this new method have excellent biological activity. Additionally, the slightly lowered inhibitory activity of **15** against thermolysin indicates that the contacts of sugar moiety with the enzyme have limited contribution to the free energy of binding.

4. Experimental

4.1. Chemistry

4.1.1. General

Chemical reagents and solvents were obtained from Acros, Aldrich, Alfa Aesar, and Beijing Chemical Works. Commercial grade reagents were used without further purification unless otherwise noted. Anhydrous solvents were obtained through standard laboratory protocols. Reactions were monitored by analytical thin-layer chromatography on plates coated with 0.25 mm silica gel 60 F254 (Qingdao Haiyang Chemicals, China). TLC plates were visualized by UV irradiation (254 nM) or stained with 20% sulfuric acid in ethanol. Flash column chromatography employed silica gel (particle size 32–63 μM, Qingdao Haiyang Chemicals, China). Size-exclusion chromatography employed Sephadex LH-20 gel. NMR spectra were obtained with a Bruker AV-400 instrument with chemical shifts reported in parts per million (ppm, δ) referenced

to $CDCl_3$ or D_2O. IR spectra were recorded on a Bruker Vertex-70 spectrometer. HPLC traces were recorded on an Agilent 1200 instrument equipped with an Eclipse XDB-C18 analytical column (4.6 × 150 mm, 5 μM; Agilent Technologies). Low-resolution and high-resolution mass spectra were obtained with a Bruker amaZon SL mass spectrometer and a Bruker Dalton micrOTOF-Q II spectrometer respectively at JXSTNU analysis center and reported as *m*/*z*.

4.1.2. Synthesis of phosphoramidion, β anomer, and intermediates

4.1.2.1. 2,3,4-Tri-*O*-acetyl-6-deoxy-α-L-mannopyranose (2). To slurry of sodium acetate (6.4 g, 78 mmol) in acetic anhydride (150 mL, 1.58 mol) was added L-rhamnose (20.0 g, 122 mmol) in four portions over 30 min. The reaction was refluxed for 2 h, and stirred at 20 °C for 2 h. The solution was poured into ice water (400 mL) and extracted with ethyl acetate (250 mL × 2). The combined organic phase was washed with saturated $NaHCO_3$ aqueous solution (300 mL), and dried over anhydrous Na_2SO_4. Concentration in vacuo afforded **6** as yellow syrup (38.1 g, 94%). To a solution of ethylenediamine (10.7 mL, 160 mmol) in THF (200 mL) was added acetic acid (9.2 mL, 160 mmol). The reaction was stirred for 30 min. A solution of **6** (38.1 g, 114 mmol) in THF (50 mL) was added, and the reaction was stirred overnight. After the solvent was removed in vacuo, the residue was dissolved in ethyl acetate (400 mL), washed with deionized H_2O (150 mL), saturated $NaHCO_3$ aqueous solution (150 mL), and 5% HCl aqueous solution (150 mL), dried with anhydrous Na_2SO_4, and concentrated in vacuo. Flash column chromatography on silica gel (petroleum ether/ethyl acetate 3:1) afforded **2** (24.1 g, 74%) as a white solid, mp: 94–95 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.29–5.25 (m, 1H), 5.15 (d, J = 2.0 Hz, 1H), 5.07 (t, J = 9.9 Hz, 1H), 4.15–4.08 (m, 1H), 3.37 (br, 1H), 2.15 (s, 3H), 2.05 (s, 3H), 1.99 (s, 3H), 1.82–1.77 (m, 1H), 1.21 (d, J = 6.2 Hz, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.3, 170.1, 92.0, 71.1, 70.2, 68.7, 66.3, 20.9, 20.8, 20.7, 17.4 ppm; IR (KBr): υ_{max} 3435, 2988, 2910, 1732, 1445, 1381, 1222, 1053, 835, 785, 692, 578 cm^{-1}; LRMS (ESI+): *m*/*z* calcd for $C_{12}H_{19}O_8$ $[M+H]^+$ 291.1; found 291.1.

4.1.2.2. Benzyl-*N*,*N*-diisopropylchlorophosphoramidite (3). To a cooled solution of PCl_3 (59.2 mL, 682 mmol) in CH_2Cl_2 (200 mL) at 0 °C was added a solution of benzyl alcohol (14.2 mL, 136 mmol) in CH_2Cl_2 (50 mL) dropwise over 3 h under an atmosphere of argon. The reaction was stirred at 20 °C for 2 h. Concentration in vacuo afforded **8** as light yellow oil (28.5 g, 99%). To a solution of **8** (28.5 g, 134 mmol) in ether (120 mL) was added a solution of diisopropylamine (19.3 mL, 136 mmol) and triethylamine (19.0 mL, 136 mmol) in ether (50 mL) dropwise under an atmosphere of argon at −20 °C over 3 h. The reaction was stirred overnight at 20 °C. The triethylammonium chloride was removed by filtration, and the filtrate was concentrated in vacuo. Vacuum distillation (90 °C, 8 mm Hg) of the residue oil afforded **3** (28.4 g, 82%) as colorless oil; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.37 (s, 4H), 7.33–7.32 (m, 5H), 4.99–4.91 (m, 2H), 3.90–3.84 (m, 2H), 1.35 (d, J = 6.0 Hz, 6H), 1.27 (d, J = 5.6 Hz, 6H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 137.5, 128.5, 127.9, 127.3, 67.6, 46.1, 46.0, 24.1, 23.3 ppm; ^{31}P NMR (161 MHz, $CDCl_3$): δ 181.57 ppm.

4.1.2.3. *N*-(*tert*-Butoxycarbonyl)-L-leucyl-L-tryptophan benzyl ester (11). To a solution of **9** (231 mg, 1 mmol) in CH_2Cl_2 (20 mL) at 0 °C was added EDC·HCl (201 mg, 1.05 mmol) and HOBt (148 mg, 1.1 mmol) under an atmosphere of argon. The reaction was stirred for 30 min. **10** (293 mg, 1 mmol) in CH_2Cl_2 (5 mL) was added dropwise and stirred overnight at 20 °C. The reaction solution was washed with saturated $NaHCO_3$ aqueous solution (20 mL × 2), dried with anhydrous Na_2SO_4, and concentrated in vacuo. Flash column chromatography on silica gel (petroleum ether/ethyl acetate 5:1) afforded **11** (456 mg, 90%) as a white solid; mp: 119–120 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.02 (s, 1H), 7.51 (d, J = 7.7 Hz, 1H), 7.34–7.32 (m, 4H), 7.21–7.16 (m, 3H), 7.09 (t, J = 7.4 Hz, 1H), 6.86 (s, 1H), 6.53 (d, 1H), 5.05 (s, 2 H), 4.94 (dd, J_1 = 5.4 Hz, J_2 = 12.7 Hz, 1H), 4.79 (d, J = 7.6 Hz, 1H), 4.07 (br, 1H), 3.32 (t, J = 3.7 Hz, 2H), 1.62–1.54 (m, 2H), 1.40 (s, 10H), 0.86 (d, J = 5.9 Hz, 6H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.1, 171.4, 155.5, 136.0, 135.1, 128.5, 128.4, 127.6, 123.0, 122.2, 119.6, 118.6, 111.2, 109.7, 79.9, 67.2, 53.1, 52.9, 41.4, 28.2, 27.6, 24.6, 22.8, 21.8 ppm; IR (KBr): υ_{max} 3377, 3335, 3284, 2961, 1720, 1665, 1521, 1456, 1364, 1275, 1166, 1047, 1020, 744, 688 cm^{-1}; HRMS (ESI+): *m*/*z* calcd for $C_{29}H_{38}N_3O_5$ $[M+H]^+$ 508.2806; found 508.2815.

4.1.2.4. L-Leucine-L-tryptophan benzyl ester (4). Compound **11** (507 mg, 1 mmol) was dissolved in a solution of trifluoroacetic acid in CH_2Cl_2 (20% v/v, 20 mL) and stirred at 20 °C for 2 h. After the solvent was removed in vacuo, the residue was dissolved in CH_2Cl_2 (30 mL), washed with saturated $NaHCO_3$ aqueous solution (20 mL × 2), dried with anhydrous Na_2SO_4, and concentrated in vacuo to afford **4** (405 mg, 99%) as a white solid. Due to the concern of potential cyclization of dipeptide,[7b] crude **4** was directly applied to the next step without further purification.

4.1.2.5. Benzyl(5*S*,8*S*)-8-(1*H*-indol-3-ylmethyl)-5-(2-methylpropyl)-6-oxo-1-phenyl-3-{[(2*S*,3*R*,4*R*,5*R*,6*S*)-3,4,5-tris(acetyloxy)-6-methyltetrahydro-2*H*-pyran-2-yl]oxy}-2-oxa-4,7-diaza-3-phosphanonan-9-oate-3-oxide (14a). General method for the synthesis of α-L-rhamnosyl-1-phosphoramidates: To a solution of **2** (580 mg, 2 mmol) in anhydrous diethyl ether (25 mL) at 0 °C were added DBU (0.50 mL, 3.4 mmol) and **3** (821 mg, 3.0 mmol) in anhydrous diethyl ether (10 mL) dropwise under an atmosphere of argon. The reaction was stirred for 30 min at 0 °C. The precipitated salt was removed by filtration, and the filtrate was concentrated in vacuo to afford crude **12**. To a solution of **12** in CH_3CN (10 mL) was added 1*H*-tetrazole (280 mg, 4 mmol) and deionized H_2O (0.1 mL, 5.6 mmol). The reaction was stirred for 30 min. After the solvent was removed in vacuo, the residue was dissolve in CH_2Cl_2 (50 mL), and washed with 2% HCl aqueous solution (30 mL × 2) and deionized H_2O (30 mL × 2). The combined organic phase was dried with anhydrous Na_2SO_4 and concentrated in vacuo to afford crude **13** as yellow syrup. To a solution of **4** (326 mg, 0.8 mmol) in CH_3CN (5 mL) was added TEA (0.21 mL, 1.5 mmol), CCl_4 (0.49 mL, 5 mmol) and a solution of crude **12** (444 mg, 1 mmol) in CH_3CN (5 mL) at 0 °C. The reaction was stirred for 30 min at 20 °C. The reaction was concentrated in vacuo and ethyl acetate (5 mL) was added to the residue. The precipitation was removed by filtration, and the filtrate was concentrated in vacuo. Flash column chromatography on silica gel (petroleum ether/ethyl acetate 2:1 to 1:1) afforded **14a** (490 mg, 72%) as a white solid, mp: 63–65 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.77, 8.40 (s, 1H), 7.49 (d, J = 7.8 Hz, 1H), 7.32–7.22 (m, 10H), 7.14 (dd, J_1 = 6.8 Hz, J_2 = 11.9 Hz, 1H), 7.08–6.92 (m, 4H), 5.61, 5.33 (d, J = 6.4 Hz, 1H), 5.33 (s, 1H), 5.27–5.23 (m, 1H), 5.10–4.88 (m, 6H), 4.02–3.94 (m, 1H), 3.79–3.74 (m, 2H), 3.33–3.29 (m, 2H), 2.22 (s, 1H), 2.10–1.97 (m, 9H), 1.71–1.53 (m, 2H), 1.39–1.34 (m, 1H), 1.17, 1.08 (d, J = 6.0 Hz, 3H), 0.89–0.82 (m, 6H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.5, 171.5, 169.8, 136.1, 135.7, 135.1, 128.5, 127.5, 123.3, 121.9, 119.3, 118.4, 111.3, 109.4, 94.5, 70.2, 69.2, 68.7, 68.4, 68.2, 67.1, 54.1, 52.7, 43.4, 27.4, 24.2, 22.7, 21.6, 20.6, 17.1 ppm; ^{31}P NMR (161 MHz, $CDCl_3$): δ 6.42, 5.46 ppm; IR (KBr): υ_{max} 3410, 3064, 2962, 1757, 1678, 1517, 1458, 1381, 1222, 1170, 1039, 960, 746, 696, 603, 495 cm^{-1}; HRMS (ESI+): *m*/*z* calcd for $C_{43}H_{53}N_3O_{13}P$ $[M+H]^+$ 850.3311; found 850.3297.

4.1.2.6. Methyl(2*S*)-2-{[(benzyloxy){[(2*S*,3*R*,4*R*,5*R*,6*S*)-3,4,5-tris(acetyloxy)-6-methyltetrahydro-2*H*-pyran-2-yl]oxy}phosphoryl]amino}propanoate (19a). Starting from L-alanine methyl ester hydrochloride (112 mg, 0.8 mmol), **19a** was synthesized according to the procedure described for **14a**. Flash column chromatography afforded **19a** (306 mg, 70%) as colorless syrup; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.39–7.31 (m, 5H), 5.58, 5.52 (d, J = 7.0 Hz, 1H), 5.29–5.23 (m, 2H), 5.09–5.04 (m, 3H), 4.07–3.90 (m, 2H), 3.72, 3.69 (s, 3H), 3.50 (dd, J_1 = 10.2 Hz, J_2 = 13.7 Hz, 1H), 2.13, 2.12 (s, 3H), 2.03 (s, 3H), 1.97 (s, 3H), 1.40, 1.32 (d, J = 7.1 Hz, 3H), 1.20, 1.14 (d, J = 6.2 Hz, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.8, 169.6, 135.7, 128.5, 128.4, 127.9, 127.7, 94.5, 70.3, 69.3, 68.7, 68.4, 68.3, 52.4, 50.0, 20.6, 17.1 ppm; ^{31}P NMR (161 MHz, $CDCl_3$): δ 5.14, 4.23 ppm; IR (KBr): υ_{max} 3629, 3211, 2592, 1747, 1446, 1377, 1222, 1020, 750, 595 cm^{-1}; HRMS (ESI+): m/z calcd for $C_{23}H_{33}NO_{12}$ $[M+H]^+$ 546.1735; found 546.1723.

4.1.2.7. Methyl(2S)-2-{[(benzyloxy){[(2S,3R,4R,5R,6S)-3,4,5-tris(acetyloxy)-6-methyltetrahydro-2H-pyran-2-yl]oxy}phosphoryl]amino}-4-methylpentanoate (20a). Starting from L-leucine methyl ester hydrochloride (145 mg, 0.8 mmol), **20a** was synthesized according to the procedure described for **14a**. Flash column chromatography afforded **20a** (325 mg, 69%) as colorless syrup; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.38–7.30 (m, 5H), 5.57, 5.50 (d, J = 6.8 Hz, 1H), 5.28–5.21 (m, 2H), 5.10–5.01 (m, 3H), 4.05–3.96 (m, 1H), 3.92–3.78 (m, 1H), 3.70, 3.66 (s, 3H), 3.43–3.34 (m, 1H), 2.11 (s, 3H), 2.02, 2.01 (s, 3H), 1.95 (s, 3H), 1.75–1.56 (m, 1H), 1.58–1.38 (m, 2H), 1.20, 1.11 (d, J = 6.2 Hz, 3H), 0.91 (t, J = 6.7 Hz, 3 H), 0.86 (t, J = 5.8 Hz, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.0, 169.7, 169.5, 135.8, 128.5, 128.4, 127.9, 127.7, 94.6, 70.3, 69.2, 68.7, 68.4, 68.1, 52.9, 52.2, 43.6, 24.3, 22.5, 21.8, 20.6, 20.5, 17.2 ppm; ^{31}P NMR (161 MHz, $CDCl_3$): δ 6.12, 4.90 ppm; IR (KBr): υ_{max} 3647, 3496, 3215, 2956, 1747, 1373, 1218, 1052, 954, 739, 603, 499 cm^{-1}; HRMS (ESI+): m/z calcd for $C_{26}H_{39}NO_{12}P$ $[M+H]^+$ 588.2204; found 588.2217.

4.1.2.8. Dimethyl(2S)-2-{[(benzyloxy){[(2S,3R,4R,5R,6S)-3,4,5-tris(acetyloxy)-6-methyltetrahydro-2H-pyran-2-yl]oxy}phosphoryl]amino}pentanedioate (21a). Starting from L-glutamic dimethyl ester hydrochloride (212 mg, 0.8 mmol), **21a** was synthesized according to the procedure described for **14a**. Flash column chromatography afforded **21a** (311 mg, 63%) as colorless syrup; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.40–7.31 (m, 5H), 5.57, 5.51 (d, J = 6.8 Hz, 1H), 5.28–5.20 (m, 2H), 5.10–5.03 (m, 3H), 4.02–3.86 (m, 2H), 3.73, 3.70 (s, 3H), 3.63, 3.62 (s, 3H), 3.58–3.53 (m, 1H), 2.44–2.35 (m, 2H), 2.12 (s, 3H), 2.03 (d, J = 3.4 Hz, 4H), 1.96 (s, 3H), 1.93–1.85 (m, 1H), 1.20, 1.12 (d, J = 6.2 Hz, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.8, 169.7, 169.5, 135.7, 128.5, 128.4, 127.9, 94.6, 70.3, 69.2, 68.8, 68.5, 68.4, 68.2, 53.6, 52.5, 51.6, 29.6, 29.3, 20.6, 17.1 ppm; ^{31}P NMR (161 MHz, $CDCl_3$): δ 5.95, 4.96 ppm; IR (KBr): υ_{max} 3649, 2954, 1741, 1452, 1373, 1220, 1046, 947, 750, 595, 502 cm^{-1}; HRMS (ESI+): m/z calcd for $C_{26}H_{37}NO_{14}P$ $[M+H]^+$ 618.1946; found 618.1958.

4.1.2.9. Methyl(2*S*)-2-{[(benzyloxy){[(2*S*,3*R*,4*R*,5*R*,6*S*)-3,4,5-tris(acetyloxy)-6-methyltetrahydro-2*H*-pyran-2-yl]oxy}phosphoryl]amino}-3-phenylpropanoate (22a). Starting from L-phenylalanine methyl ester hydrochloride (133 mg, 0.8 mmol), **22a** was synthesized according to the procedure described for **14a**. Flash column chromatography afforded **22a** (333 mg, 67%) as colorless syrup; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.35–7.11 (m, 10H), 5.50, 5.42 (d, J = 6.7 Hz, 1H), 5.26–5.19 (m, 2H), 5.05 (t, J = 10.0 Hz, 1H), 4.98–4.93 (m, 1H), 4.89–4.68 (m, 1H), 4.28–4.05 (m, 1H), 4.01–3.95 (m, 1H), 3.71, 3.66 (s, 3H), 3.36, 3.28 (t, J = 10.6 Hz, 1H), 3.05, 2.94 (t, J = 5.1 Hz, 2H), 2.14, 2.13 (s, 3H), 2.04, 2.03 (s, 3H), 1.98 (s, 3H), 1.19, 1.12 (d, J = 6.2 Hz, 3H) ppm; ^{13}C NMR (400 MHz, $CDCl_3$): δ 172.6, 169.8, 169.6, 135.7, 129.4, 128.6, 128.4, 127.8, 127.7, 127.1, 94.5, 70.3, 69.2, 68.4, 68.3, 68.2, 55.6, 55.2, 40.3, 20.6, 17.1 ppm; ^{31}P NMR (161 MHz, $CDCl_3$): δ 5.44, 4.36 ppm; IR (KBr): υ_{max} 3469, 3049, 2952, 1749, 1517, 1441, 1371, 1220, 1052, 960, 744, 700, 593, 502 cm^{-1}; HRMS (ESI+): m/z calcd for $C_{29}H_{37}NO_{12}P$ $[M+H]^+$ 622.2048; found 622.2035.

4.1.2.10. Methyl(2*S*)-2-{[(benzyloxy){[(2*S*,3*R*,4*R*,5*R*,6*S*)-3,4,5-tris(acetyloxy)-6-methyltetrahydro-2*H*-pyran-2-yl]oxy}phosphoryl]amino}-3-(1*H*-indol-3-yl)propanoate (23a). Starting from L-tryptophan methyl ester hydrochloride (204 mg, 0.8 mmol), **23a** was synthesized according to the procedure described for **14a**. Flash column chromatography afforded **23a** (360 mg, 68%) as colorless syrup; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.58 (s, 1H), 7.51 (d, J = 7.6 Hz, 1H), 7.33–7.25 (m, 6H), 7.16–7.04 (m, 3H), 5.55 (d, J = 6.6 Hz, 1H), 5.26–5.19 (m, 2H), 5.07–4.95 (m, 2H), 4.91–4.86 (m, 1H), 4.28–4.24 (m, 1H), 3.93–3.88 (m, 1H), 3.60 (s, 3H), 3.52 (t, J = 10.7 Hz, 1H), 3.24 (d, J = 5.4 Hz, 2H), 2.12 (s, 3H), 2.03 (s, 3H), 1.98 (s, 3H), 1.07 (d, J = 6.2 Hz, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.0, 169.9, 169.8, 169.7, 136.1, 135.9, 128.5, 128.3, 127.6, 127.5, 123.4, 121.9, 119.4, 118.4, 111.3, 109.3, 94.5, 70.3, 69.3, 68.5, 68.3, 68.2, 54.8, 52.3, 29.9, 20.7, 20.6, 17.1 ppm; ^{31}P NMR (161 MHz, $CDCl_3$): δ 5.69 ppm; IR (KBr): υ_{max} 3293, 2932, 1749, 1434, 1368, 1215, 1151, 1035, 970, 744, 682, 600 cm^{-1}; HRMS (ESI+): m/z calcd for $C_{31}H_{38}N_2O_{12}P$ $[M+H]^+$ 661.2157; found 661.2142.

4.1.2.11. (2*S*,3*R*,4*R*,5*R*,6*S*)-2-{[(Benzyloxy)(dodecylamino)phosphoryl]oxy}-6-methyltetrahydro-2*H*-pyran-3,4,5-triyltriacetate (24a). Starting from dodecylamine (177 mg, 0.8 mmol), **24a** was synthesized according to the procedure described for **14a**. Flash column chromatography afforded **24a** (352 mg, 70%) as colorless syrup; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.40–7.30 (m, 5H), 5.57, 5.53 (d, J = 7.2 Hz, 1H), 5.31–5.25 (m, 2H), 5.10–5.05 (m, 3H), 4.07–3.96 (m, 1H), 2.94–2.84 (m, 2H), 2.75–2.70 (m, 1H), 2.14 (s, 3H), 2.04, 2.03 (s, 3H), 1.98 (s, 3H), 1.45–1.41 (m, 2H), 1.23–1.13 (m, 21H), 0.86 (t, J = 6.4 Hz, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.8, 136.0, 128.5, 128.4, 127.8, 127.7, 94.3, 70.4, 69.4, 68.4, 68.3, 68.1, 41.4, 31.8, 31.6, 29.6, 29.3, 29.2, 26.5, 22.6, 20.7, 17.3, 14.1 ppm; ^{31}P NMR (161 MHz, $CDCl_3$): δ 6.93, 6.12 ppm; IR (KBr): υ_{max} 3475, 3255, 2925, 2858, 1753, 1462, 1371, 1220, 1153, 2049, 960, 740, 597, 507 cm^{-1}; HRMS (ESI+): m/z calcd for $C_{31}H_{51}NO_{10}P$ $[M+H]^+$ 628.3245; found 628.3258.

4.1.2.12. (2*S*,3*R*,4*R*,5*R*,6*S*)-2-{[(Benzyloxy)(morpholin-4-yl)phosphoryl]oxy}-6-methyltetrahydro-2*H*-pyran-3,4,5-triyltriacetate (25a). Starting from morpholine (70 mg, 0.8 mmol), **25a** was synthesized according to the procedure described for **14a**. Flash column chromatography afforded **25a** (297 mg, 70%) as colorless syrup; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.39–7.33 (m, 5H), 5.56, 5.52 (d, J = 7.0 Hz, 1H), 5.32–5.19 (m, 2H), 5.11–5.03 (m, 3H), 4.04–3.87 (m, 1H), 3.60–3.53 (m, 4H), 3.14–3.05 (m, 4H), 2.13, 2.12 (s, 3H), 2.03, 2.02 (s, 3H), 1.97 (s, 3H), 1.23, 1.13 (d, J = 6.2 Hz, 3H) ppm; ^{13}C NMR (400 MHz, $CDCl_3$): δ 169.9, 169.7, 169.6, 135.7, 128.6, 128.0, 127.8, 94.3, 70.2, 69.3, 68.7, 68.5, 68.3, 66.7, 44.4, 20.6, 17.2 ppm; ^{31}P NMR (161 MHz, $CDCl_3$): δ 6.12, 5.26 ppm; IR (KBr): υ_{max} 3642, 3483, 2976, 2863, 1749, 1447, 1373, 1218, 1060, 958, 704, 606, 482 cm^{-1}; HRMS (ESI+): m/z calcd for $C_{23}H_{33}NO_{11}P$ $[M+H]^+$ 530.1786; found 530.1772.

4.1.2.13. Benzyl(5*S*,8*S*)-8-(1*H*-indol-3-ylmethyl)-5-(2-methylpropyl)-6-oxo-1-phenyl-3-{[(2*R*,3*R*,4*R*,5*R*,6*S*)-3,4,5-tris(acetyloxy)-6-methyltetrahydro-2*H*-pyran-2-yl]oxy}-2-oxa-4,7-diaza-3-phosphanonan-9-oate-3-oxide (14b). General method for the synthesis of β-L-rhamnosyl-1-phosphoramidates: To a solution of **2** (580 mg, 2.0 mmol) in anhydrous CH_2Cl_2 (3.0 mL) at 35 °C was added Et_3N (0.47 mL, 3.4 mmol) and a solution of **3** (821 mg, 3.0 mmol) in CH_2Cl_2 (1.4 mL) dropwise under an atmosphere of dry argon. The reaction was stirred for 30 min at 35 °C. The precipitated salt was removed by filtration, and the filtrate was concentrated in vacuo to afford crude **12**. The acid-catalyzed hydrolysis and oxidative coupling were performed according to the procedures described for **14a**. Flash column chromatography (petroleum ether/ethyl acetate 3:1 to 2:1) afforded **14b** (360 mg, 53%) as a white solid; mp: 64–66 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.71, 8.55 (s, 1H), 7.49, 7.47 (d, J = 7.8 Hz, 1H), 7.32–7.19 (m, 10H), 7.14–6.90 (m, 4H), 5.49–5.31 (m, 2H), 5.06 (s, 1H), 5.05–4.83 (m, 6H), 3.76–3.64 (m, 2H), 3.55–3.36 (m, 3H), 2.33 (s, 1H), 2.10, 2.06 (s, 3H), 2.03 (s, 3H), 1.99, 1.96 (s, 3H), 1.70–1.49 (m, 2H), 1.38–1.29 (m, 1H), 1.21, 1.14 (d, J = 6.1 Hz, 3H), 0.82–0.76 (m, 6H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.6, 171.6, 170.3, 169.9, 136.1, 135.8, 135.3, 128.4, 127.7, 123.4, 121.9, 119.4, 118.5, 111.4, 109.5, 93.5, 71.1, 70.6, 70.2, 69.2, 68.6, 67.1, 53.9, 52.9, 43.3, 27.5, 24.2, 22.7, 21.8, 20.6, 17.2 ppm; ^{31}P NMR (161 MHz, $CDCl_3$) δ 6.49 ppm; IR (KBr): υ_{max} 3413, 3066, 2953, 1752, 1661, 1520, 1460, 1368, 1215, 1046, 925, 739, 598, 496 cm^{-1}; HRMS (ESI+): m/z calcd for $C_{43}H_{53}N_3O_{13}P$ $[M+H]^+$ 850.3311; found 850.3325.

4.1.2.14. Methyl(2*S*)-2-{[(benzyloxy){[(2*R*,3*R*,4*R*,5*R*,6*S*)-3,4,5-tris(acetyloxy)-6-methyltetrahydro-2*H*-pyran-2-yl]oxy}phosphoryl]amino}propanoate (19b). Starting from L-alanine methyl ester hydrochloride (112 mg, 0.8 mmol), **19b** was synthesized according to the procedure described for **14b**. Flash column chromatography afforded **19b** (209 mg, 48%) as colorless syrup; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.39–7.26 (m, 5H), 5.58, 5.52 (d, J = 6.8 Hz, 1H), 5.27–5.23 (m, 2H), 5.09–5.03 (m, 3H), 4.0–3.90 (m, 2H), 3.72, 3.69 (s, 3H), 3.54 (t, J = 9.6 Hz, 1H), 2.12 (s, 3H), 2.03 (s, 3H), 1.96 (s, 3H), 1.39, 1.32 (d, J = 7.2 Hz, 3H), 1.19, 1.14 (d, J = 6.2 Hz, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.9, 169.2, 169.0, 168.7, 134.9, 127.5, 126.8, 126.7, 92.4, 70.1, 69.5, 69.0, 68.1, 67.5, 51.4, 48.7, 19.7, 19.5, 16.2 ppm; ^{31}P NMR (161MHz, $CDCl_3$): δ 5.53 ppm; IR (KBr): υ_{max} 3344, 2985, 1747, 1444, 1373, 1218, 1056, 739, 592, 505 cm^{-1}; HRMS (ESI+): m/z calcd for $C_{23}H_{33}NO_{12}P$ $[M+H]^+$ 546.1735; found 546.1749.

4.1.2.15. Methyl(2*S*)-2-{[(benzyloxy){[(2*R*,3*R*,4*R*,5*R*,6*S*)-3,4,5-tris(acetyloxy)-6-methyltetrahydro-2*H*-pyran-2-yl]oxy}phosphoryl]amino}-4-methylpentanoate (20b). Starting from L-leucine methyl ester hydrochloride (145 mg, 0.8 mmol), **20b** was synthesized according to the procedure described for **14b**. Flash column chromatography afforded **20b** (230 mg, 49%) as colorless syrup; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.37–7.31 (m, 5H), 5.58, 5.50 (d, J = 6.84, 1H), 5.27–5.22 (m, 2H), 5.09–5.03 (m, 3H), 4.04–3.97 (m, 1H), 3.92–3.80 (m, 1H), 3.72, 3.68 (s, 3H), 3.26 (dd, J_1 = 10.4 Hz, J_2 = 17.9 Hz, 1H), 2.13 (s, 3H), 2.03 (s, 3 H), 1.97 (s, 3H), 1.76–1.67 (m, 1H), 1.56–1.38 (m, 2H), 1.21, 1.12 (d, J = 6.1 Hz, 3H), 0.91–0.85 (m, 6H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.0, 169.8, 169.7, 169.6, 135.8, 128.5, 128.4, 127.9, 127.7, 94.6, 70.3, 69.2, 68.7, 68.4, 68.1, 52.9, 52.2, 43.7, 24.4, 22.6, 21.8, 20.7, 20.5, 17.5 ppm; ^{31}P NMR (161 MHz, $CDCl_3$): δ 6.34 ppm; IR (KBr): υ_{max} 3631, 3492, 3211, 1747, 1460, 1373, 1218, 1055, 957, 743, 607, 460 cm^{-1}; HRMS (ESI+): m/z calcd for $C_{26}H_{39}NO_{12}P$ $[M+H]^+$ 588.2204; found 588.2213.

4.1.2.16. Dimethyl (2*S*)-2-{[(benzyloxy){[(2*R*,3*R*,4*R*,5*R*,6*S*)-3,4,5-tris(acetyloxy)-6-methyltetrahydro-2*H*-pyran-2-yl]oxy}phosphoryl]amino}pentanedioate (21b). Starting from L-glutamic dimethyl hydrochloride (212 mg, 0.8 mmol), **21b** was synthesized according to the procedure described for **14b**. Flash column chromatography afforded **21b** (237 mg, 48%) as colorless syrup; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.34–7.25 (m, 5H), 5.46–5.37 (m, 2H), 5.08–4.92 (m, 4H), 3.90–3.81 (m, 1H), 3.68 (s, 3H), 3.63 (d, J = 7.6 Hz, 2H), 3.59 (s, 3H), 2.43–2.30 (m, 2H), 2.13 (s, 3H), 2.07 (s, 1H), 2.02 (s, 3H), 1.95 (s, 3H), 1.90–1.79 (m, 1H), 1.25, 1.23 (d, J = 6.1 Hz, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.9, 170.2, 169.8, 135.8, 128.5, 128.4, 127.7, 93.5, 71.1, 70.5, 70.0, 69.0, 68.6, 53.3, 52.5, 51.6, 29.4, 29.1, 20.6, 17.2 ppm; ^{31}P NMR (161 MHz, $CDCl_3$): δ 5.92 ppm; IR (KBr): υ_{max} 3647, 3467, 2952, 1741, 1467, 1436, 1373, 1220, 1056, 928, 744, 595, 505 cm^{-1}; HRMS (ESI+): m/z calcd for $C_{26}H_{37}NO_{14}P$ $[M+H]^+$ 618.1946; found 618.1933.

4.1.2.17. Methyl(2*S*)-2-{[(benzyloxy){[(2*R*,3*R*,4*R*,5*R*,6*S*)-3,4,5-tris(acetyloxy)-6-methyltetrahydro-2*H*-pyran-2-yl]oxy}phosphoryl]amino}-3-phenylpropanoate (22b). Starting from L-phenylalaine methyl ester hydrochloride (133 mg, 0.8 mmol), **22b** was synthesized according to the procedure described for **14b**. Flash column chromatography afforded **22b** (224 mg, 45%) as colorless syrup; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.35–7.08 (m, 10H), 5.49–5.18 (m, 2H), 5.06–4.78 (m, 4H), 4.26–4.13 (m, 1H), 3.68, 3.62 (s, 3H), 3.54–3.38 (m, 2H), 3.09–2.90 (m, 2H), 2.13 (d, J = 6.7 Hz, 3H), 2.05 (s, 3H), 1.98 (s, 3H), 1.23 (d, J = 6.1 Hz, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.4, 170.3, 170.1, 169.8, 135.8, 129.5, 129.4, 128.5, 128.3, 127.8, 127.7, 127.1, 93.4, 71.2, 70.5, 70.1, 69.1, 68.4, 55.2, 52.3, 40.3, 20.7, 17.2 ppm; ^{31}P NMR (161 MHz, $CDCl_3$): δ 5.62 ppm; IR (KBr): υ_{max} 3479, 2979, 1749, 1446, 1373, 1220, 1029, 869, 747, 691, 600, 490 cm^{-1}; HRMS (ESI+): m/z calcd for $C_{29}H_{37}NO_{12}P$ $[M+H]^+$ 622.2048; found 622.2061.

4.1.2.18. Methyl(2*S*)-2-{[(benzyloxy){[(2*R*,3*R*,4*R*,5*R*,6*S*)-3,4,5-tris(acetyloxy)-6-methyltetrahydro-2*H*-pyran-2-yl]oxy}phosphoryl]amino}-3-(1*H*-indol-3-yl)propanoate (23b). Starting from L-tryptophan methyl ester hydrochloride (204 mg, 0.8 mmol), **23b** was synthesized according to the procedure described for **14b**. Flash column chromatography afforded **23b** (248 mg, 47%) as colorless syrup; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.62, 8.47 (s, 1H), 7.56, 7.53 (d, J = 7.8 Hz, 1H), 7.38–7.26 (m, 6H), 7.19–6.99 (m, 3H), 5.50–4.82 (m, 6H), 4.32–4.26 (m, 1H), 3.72–3.06 (m, 7H), 2.12, 2.10 (s, 3H), 2.06, 2.05 (s, 3H), 2.01, 1.99 (s, 3H), 1.16 (d, J = 6.1 Hz, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.1, 170.4, 170.1, 169.8, 136.0, 135.9, 128.5, 128.3, 127.8, 127.2, 123.5, 122.0, 119.5, 118.7, 111.3, 109.7, 93.5, 71.1, 70.9, 70.7, 70.5, 70.0, 69.0, 68.3, 54.5, 52.4, 30.2, 20.7, 20.6, 17.2 ppm; ^{31}P NMR (161 MHz, $CDCl_3$): δ 6.38, 5.87 ppm; IR (KBr): υ_{max} 3348, 2947, 1753, 1460, 1379, 1232, 1055, 927, 875, 740, 592 cm^{-1}; HRMS (ESI+): m/z calcd for $C_{31}H_{38}N_2O_{12}P$ $[M+H]^+$ 661.2157; found 661.2148.

4.1.2.19. (2*R*,3*R*,4*R*,5*R*,6*S*)-2-{[(Benzyloxy)(dodecylamino)phosphoryl]oxy}-6-methyltetrahydro-2*H*-pyran-3,4,5-triyltriacetate (24b). Starting from dodecylamine (177 mg, 0.8 mmol), **24b** was synthesized according to the procedure described for **14b**. Flash column chromatography afforded **24b** (226 mg, 45%) as colorless syrup; ^{1}H NMR (400 MHz, $CDCl_3$); δ 7.35–7.25 (m, 5H), 5.46 (d, J = 14.4 Hz, 1H), 5.40 (t, J = 8.2 Hz, 1H), 5.06–5.01 (m, 3H), 4.99–4.92 (m, 1H), 3.61–3.56 (m, 1H), 2.88–2.81 (m, 3H), 2.13, 2.10 (s, 3H), 2.01 (s, 3H), 1.95 (s, 3H), 1.39 (d, J = 5.6 Hz, 2H), 1.25–1.20 (m, 21H), 0.84 (t, J = 6.6 Hz, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.1, 169.9, 169.6, 136.0, 128.4, 128.3, 128.1, 127.7, 127.6, 93.4, 71.0, 70.5, 70.1, 69.2, 68.2, 41.2, 31.7, 31.4, 29.4, 29.1, 26.4, 22.5, 20.5, 17.3, 13.9 ppm; ^{31}P NMR (161 MHz, $CDCl_3$): δ 8.33 ppm; IR (KBr): υ_{max} 3479, 3240, 2927, 2856, 1755, 1460, 1373, 1220,

1163, 1049, 970, 732, 599, 493 cm^{-1}; HRMS (ESI+): *m*/*z* calcd for $C_{31}H_{51}NO_{10}P$ $[M+H]^+$ 628.3245; found 628.3257.

4.1.2.20. (2*R*,3*R*,4*R*,5*R*,6*S*)-2-{[(Benzyloxy)(morpholin-4-yl)phosphoryl]oxy}-6-methyltetrahydro-2*H*-pyran-3,4,5-triyltriacetate (25b). Starting from morpholine (0.07 mL, 0.8 mmol), **25b** was synthesized according to the procedure described for **14b**. Flash column chromatography afforded **25b** (203 mg, 48%) as colorless syrup; ^1H NMR (400 MHz, $CDCl_3$): δ 7.37–7.32 (m, 5H), 5.50, 5.49 (d, J = 11.6 Hz, 1H), 5.44, 5.38 (d, J = 8.4 Hz, 1H), 5.11–4.92 (m, 4H), 3.64–3.61 (m, 1H), 3.58–3.52 (m, 4H), 3.09–3.02 (m, 4H), 2.16, 2.13 (s, 3H), 2.05 (s, 3H), 1.98 (s, 3H), 1.28 (t, J = 6.0 Hz, 3H) ppm; ^{13}C NMR (400 MHz, $CDCl_3$): δ 170.0, 169.9, 169.7, 135.8, 128.5, 127.9, 93.5, 71.2, 70.6, 70.1, 69.2, 68.5, 66.6, 44.4, 20.6, 17.4 ppm; ^{31}P NMR (161 MHz, $CDCl_3$): δ 6.30, 5.77 ppm; IR (KBr): υ_{max} 3473, 2978, 2863, 2922, 1749, 1642, 1469, 1373, 1218, 1157, 1060, 956, 704, 607, 489 cm^{-1}; HRMS (ESI+): *m*/*z* calcd for $C_{23}H_{33}NO_{11}P$ $[M+H]^+$ 530.1786; found 530.1796.

4.1.2.21. (2*S*)-2-({(2*S*)-2-[(Hydroxy{[(2*S*,3*R*,4*R*,5*R*,6*S*)-3,4,5-trihydroxy-6-methyltetrahydro-2*H*-pyran-2-yl]oxy}phosphoryl)amino]-4-methylpentanoyl}amino)-3-(1*H*-indol-3-yl)propionate, disodium salt (1, phosphoramidon). To a solution of **14a** (85 mg, 0.1 mmol) in anhydrous MeOH (20 mL) was added 5% Pd/C (15 mg). The reaction was stirred under an atmosphere of hydrogen for 3 h at 20 °C. Then, NaOMe (32 mg, 0.6 mmol) in anhydrous MeOH (6 mL) was added to the solution dropwise at 0 °C and stirred for 3 h at 20 °C. The pH value of the reaction solution was adjusted to 7.5 by adding a solution of aqueous HCl in MeOH (0.12 M). Removal of Pd/C by filtration and concentration in vacuo afforded crude **1** as a white solid. Size-exclusion chromatography (Sephadex LH-20, deionized H_2O, 2.5 × 70 cm) afforded **1** (54 mg, 92%) as disodium salt, a white solid; mp: 176–179 °C [Commercial phosphoramidon (Sigma) from a microbial source, mp: 173–178 °C]; $[\alpha]^{20}_D$ = −17.86° (*c* = 1.0, H_2O) [Lit. $[\alpha]^{20}_D$ = −15.7° (H_2O) for di-dicyclohexylammonium salt][1a]; ^1H NMR (400 MHz, D_2O): δ 7.68 (d, J = 7.8 Hz, 1H), 7.50 (d, J = 8.0 Hz, 1H), 7.26–7.15 (m, 3H), 5.26 (d, J = 7.8 Hz, 1H), 4.54 (t, J = 6.0 Hz, 1H), 3.85–3.77 (m, 3H), 3.49–3.38 (m, 3H), 3.20, 3.16 (d, J = 7.6 Hz, 1H), 1.53 (br, 1H), 1.27 (d, J = 5.8 Hz, 3H), 1.17–1.11 (m, 1H), 0.95–0.88 (m, 1H), 0.83 (d, J = 6.5 Hz, 3H), 0.79 (d, J = 6.5 Hz, 3H) ppm; ^{13}C NMR (100 MHz, D_2O): δ 178.5, 177.1, 136.3, 127.3, 124.5, 121.9, 119.3, 119.0, 111.9, 110.4, 95.5, 72.4, 71.3, 70.0, 69.6, 55.8, 54.9, 43.2, 27.9, 24.0, 22.6, 21.2, 17.1 ppm; ^{31}P NMR (161 MHz, D_2O): δ 4.06 ppm; IR (KBr): υ_{max} 3508, 3425, 3383, 3278, 2952, 1166, 1579, 1516, 1402, 1234, 1101, 1047, 835, 746, 586, 526, 497 cm^{-1}; HRMS (ESI−): *m*/*z* calcd for $C_{23}H_{33}N_3O_{10}P$ $[M-H]^-$ 542.1909; found 542.1921.

4.1.2.22. (2*S*)-2-({(2*S*)-2-[(Hydroxy{[(2*R*,3*R*,4*R*,5*R*,6*S*)-3,4,5-trihydroxy-6-methyltetrahydro-2*H*-pyran-2-yl]oxy}phosphoryl)amino]-4-methylpentanoyl}amino)-3-(1*H*-indol-3-yl)propionate, disodium salt (15). Starting from **14b** (85 mg, 0.1 mmol), compound **15** was synthesized according to the procedure described for **1**. Recrystallization from ethyl acetate/MeOH/H_2O afforded **15** (55 mg, 93%) as disodium salt, a white solid; mp: 204–206 °C; $[\alpha]^{20}_D$ = −4.88° (c = 1.0, H_2O); ^1H NMR (400 MHz, D_2O): δ 7.72 (d, J = 7.2 Hz, 1H), 7.52 (d, J = 7.6Hz, 1H), 7.25–7.19 (m, 3H), 4.99 (d, J = 8.0 Hz, 1H), 4.56 (s, 1H), 3.88 (s, 1H), 3.59–3.38 (m, 5H), 3.23–3.18 (m, 1H), 1.54 (s, 1H), 1.30 (s, 3H), 1.20 (s, 1H), 1.00 (s, 1H), 0.83, 0.79 (d, J = 5.2 Hz, 6H) ppm; ^{13}C NMR (100 MHz, D_2O): δ 178.4, 176.9, 136.2, 127.7, 124.4, 121.8, 119.2, 119.0, 111.9, 110.3, 94.9, 72.6, 72.5, 72.0, 71.5, 55.8, 54.8, 43.3, 27.8, 23.9, 22.5, 21.4, 17.0 ppm; ^{31}P NMR (161 MHz, D_2O): δ 4.90 ppm; IR (KBr): υ_{max} 3504, 3417, 3381, 3282, 2956, 1662, 1579, 1519, 1404, 1236, 1099, 1051, 931, 883, 837, 742, 686, 587, 526, 491 cm^{-1}; HRMS (ESI−): *m*/*z* calcd for $C_{23}H_{33}N_3O_{10}P$ $[M-H]^-$ 542.1909; found 542.1916.

4.2. Enzymatic assays

4.2.1. Determination of the inhibitor constants (K_i) of 1 and 15

The K_i values were determined in a 50 mM Tris–HCl, 10 mM $CaCl_2$ buffer (pH 7.5) with FA-Gly-Leu-NH_2 (FAGLA, Bachem) as a substrate by using an Agilent 8453 UV–vis spectrophotometer in triplicate. Henderson plots were employed for the calculation of K_i values. A mixture of buffer (970 μL), inhibitor (0–80 nM, 20 μL), and thermolysin (Sigma, 40 nM, 10 μL) was incubated at 25 °C for 15 min in a cuvette (2 mL). A solution of FAGLA (0.1–0.5 mM, 1.0 mL) in Tris buffer pH 7.5 was added into the cuvette. The absorbance decrease upon cleavage of FAGLA by thermolysin was recorded at 340 nm wavelength for 5 min. The concentration of thermolysin was determined from the molar extinction coefficient ε_{278} = 66,300. The K_i values of **1** and **15** were determined according to Eq. 1. When E_0 was fixed, dose-response measurements were repeated at increasing concentrations of A_t. The slopes of plots of $I_t/(1-\upsilon_i/\upsilon_0)$ against υ_0/υ_i increases for this competitive binding. Replots of the slopes against A_t were linear for competitive binding, and the extrapolated intercepts on the ordinate yielded the K_i values.

$$I_t/(1-\upsilon_i/\upsilon_0) = E_t + K_i[(A_t+K_a)/K_a]\upsilon_0/\upsilon_i \quad (1)$$

4.2.2. Determination of the dissociation constants (K_d) of the E-1 and E-15 complexes

The fluorescence spectra of thermolysin (E), **1**, **15**, and their binding complexes at the same molar concentration were recorded with a Hitachi F-4600 fluorescence spectrophotometer with λ_{ex} = 280 nm. To a solution of thermolysin (1.15×10^{-6} M, 980 μL) in Tris buffer pH 7.5 in a cuvette was added a solution of the inhibitor (1.15×10^{-4} M, 2 μL) in Tris buffer pH 7.5. The mixed solution was incubated at 25 °C for 15 min. The percentage of the increment of fluorescence intensity relative to the total fluorescence intensity ($\Delta F/(F_E+F_I)$) was recorded at 360 nm. The titration was repeated by adding a solution of the inhibitor (2 μL) until the total fluorescence intensity remained constant. The values of Δf and K_d were determined as adjustable parameters by the nonlinear least-squares method according to Eqs. (2)–(4) in triplicate.

$$\Delta F = \frac{1}{2}([E]_0+[I]_0+K_d-\sqrt{([E]_0+[I]_0+K_d)^2-4[E]_0[I]_0})\Delta f \quad (2)$$

$$\Delta F = F_{EI}-F_E-F_I \quad (3)$$

$$\Delta F_{max} = \Delta f[E]_0 \quad (4)$$

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.07.052.

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