DOI 10.1007/s10593-020-02704-6

Chemistry of Heterocyclic Compounds **2020**, *56*(5), 586–602

Aliphatic chain-containing macrocycles as diazonamide A analogs

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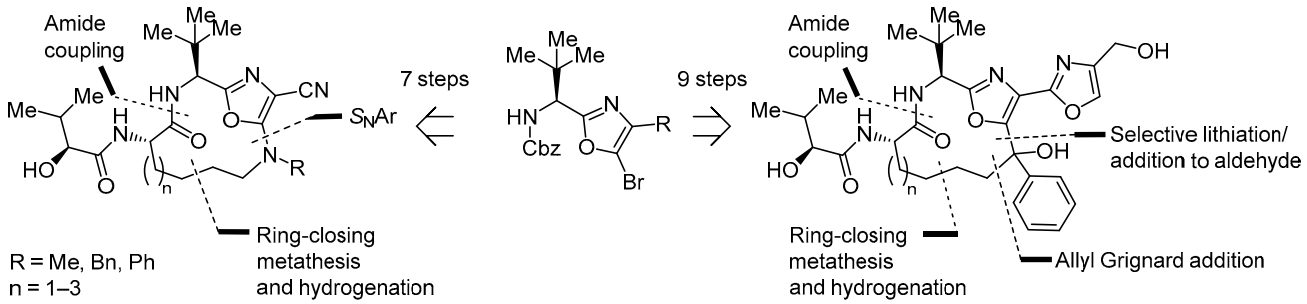
Published in Khimiya Geterotsiklicheskikh Soedinenii, 2020, *56*(5), 586–602

Submitted December 30, 2019
Accepted after revision April 3, 2020

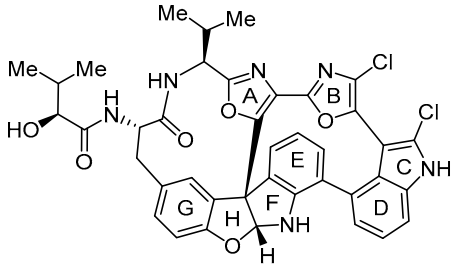


Aliphatic alkyl chain-containing 12–14-membered macrocycles have been designed as structural analogs of antimitotic natural product diazonamide A. Macrocycles were synthesized from 5-bromooxazole in 7 to 9 linear steps using Ru-catalyzed ring-closing metathesis as the key transformation. Heat effect of binding to α,β-tubulin tetramer (T4-RB3 complex) has been measured for the synthesized macrocycles by isothermal titration calorimetry method.

Keywords: diazonamide A, anticancer agents, macrocycles, ring-closing metathesis.

Chemotherapeutics are the most efficient means for the treatment of metastatic tumors. However, a majority of cancer chemotherapeutic agents cause side effects such as nausea, vomiting, diarrhea, hair loss, and pain. These side effects result from undesired cytotoxicity toward normal cells.[1] A notable exception among cancer chemotherapeutic agents is marine metabolite diazonamide A (**1**) (Fig. 1), which does not cause systemic toxicity typical for other antimitotic drugs, while exerting nanomolar cytotoxicity against a range of human tumor cell lines.[2] The greatly reduced systemic toxicity renders diazonamide A (**1**) a highly attractive cancer chemotherapeutic agent. Unfortunately, highly complex structure of the natural product **1** is an important hurdle for its use in the cancer treatment. The decrease of the structural complexity of diazonamide A (**1**) without affecting the anticancer activity is possible, as was demonstrated by the development of anticancer agent DZ-2384 (**2**) (Fig. 1).[3] It has been shown that both natural product **1** and synthetic analog **2** are microtubule-targeting agents.[2,3]

The structurally simplified diazonamide A analog **2** lacks CD subunit and heteroaromatic macrocycle as compared to the parent natural product **1** (Fig. 1). Considering further structural modifications, the substitution of the difficult to synthesize tetracyclic subunit EFGH by less complex linker is highly attractive from the synthetic viewpoint. To verify



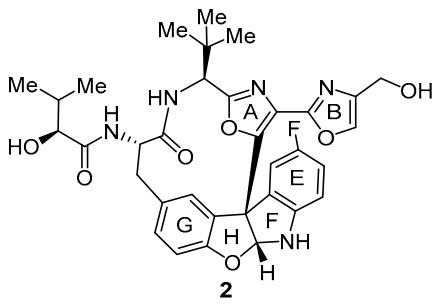


Figure 1. Antimitotic diazonamide A (**1**) and the structurally simplified synthetic analog **2**.

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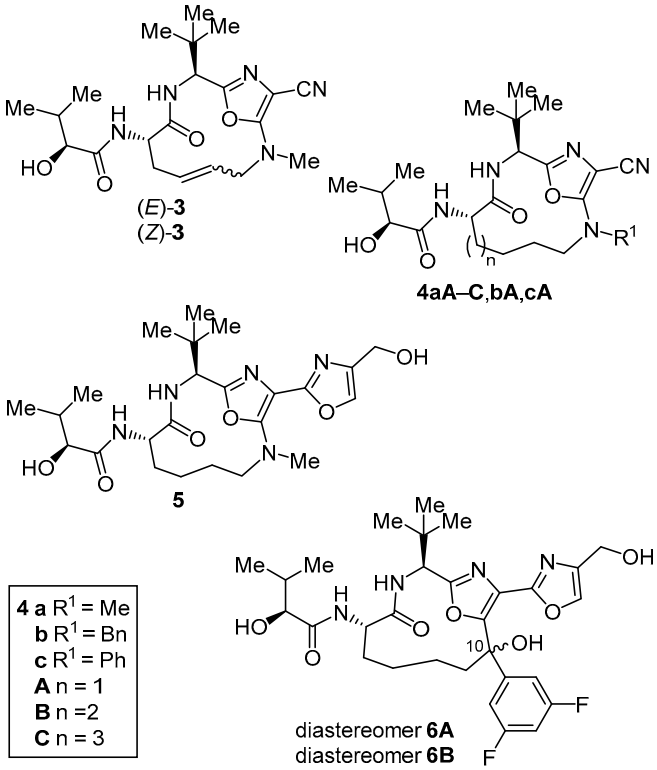


Figure 2. Structure of diazonamide A analogs **3**–**6**.

the importance of the tetracyclic subunit EFGH for the binding of cytotoxic agents **1**, **2** to tubulin, a series of diazonamide analogs **3**–**6** that feature the replacement of the EFGH subunit by aliphatic chain of various length were designed and synthesized (Fig. 2). Binding energy of the synthesized analogs **3**–**6** with tubulin has been measured by isothermal titration calorimetry (ITC) assay.

The retrosynthetic analysis of diazonamide A analogs **3**–**6** is depicted in Figure 3. We envisioned that macrocycles **7** could be constructed from the parent bisalkene intermediates **8** using the ring-closing metathesis (RCM) as the key step. The allyl moieties in the key intermediates **8** (X = NR^2) could be installed by amide coupling with chiral unsaturated amino acids and by the C–N bond formation in the S_NAr-type reaction of bromooxazole **10** and allyl amines. In addition, the allyl moiety could be also attached to the oxazole subunit in the key intermediate **8** (X = C(OH)Ar) by the nucleophilic addition of allyl metal species to ketone **11**. The latter is accessible by regioselective lithiation of bioxazole **12**,[4] followed by the *in situ* addition of lithiated species to aryl ketones.

The synthesis of macrocycles **4**–**6** commenced by the preparation of bromooxazole **10** and amino acids **16B** and **16C** using literature methods (Scheme 1). Thus, bromooxazole **10** was synthesized from the commercially available Cbz-protected (*S*)-*tert*-leucine **13** and 2-aminomalononitrile tosylate **14**, followed by amine-bromide exchange in compound **15** under the Sandmeyer reaction conditions.[5] The synthesis of unsaturated amino acid **16C** started with the formation of ester **17A** from the corresponding acid **16A**. Subsequent reaction sequence included the hydroboration of ester **17A** with 9-BBN, which was followed by Suzuki cross coupling between the *in situ* generated

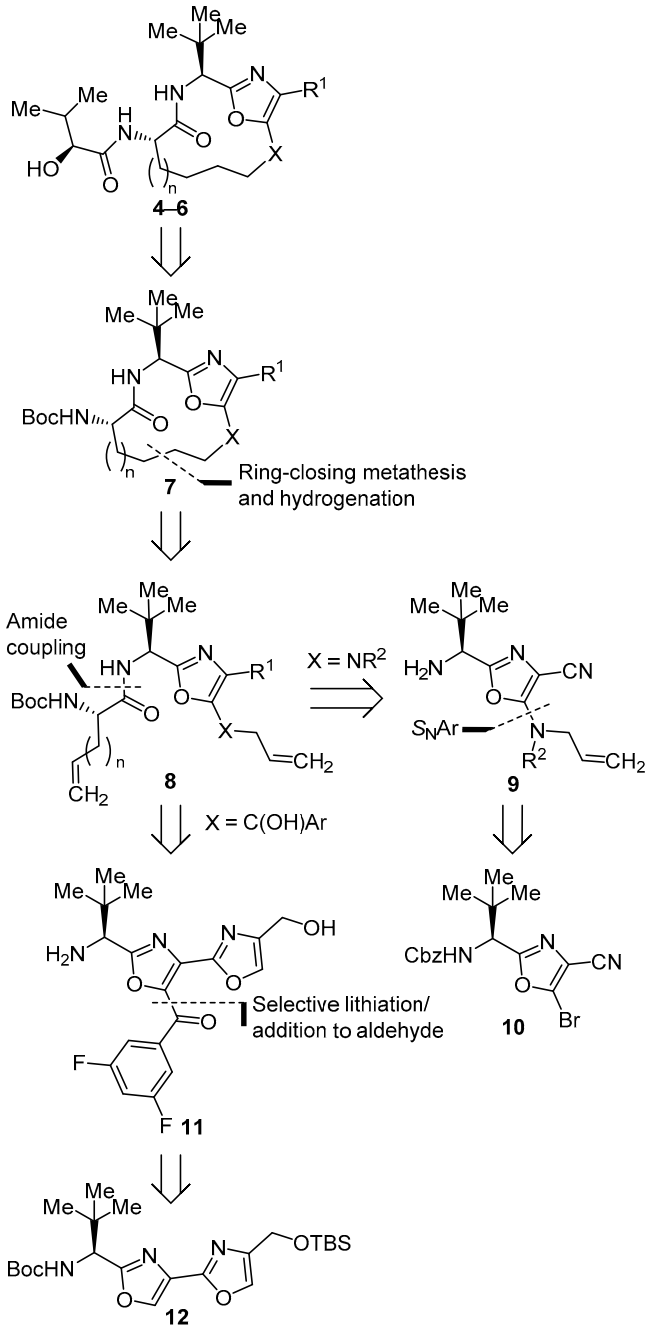
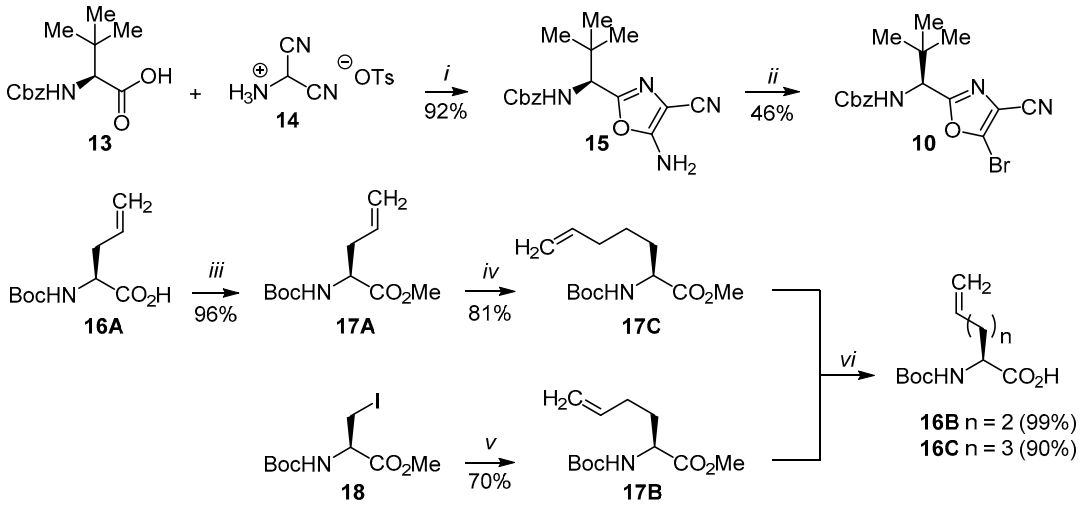


Figure 3. Retrosynthetic analysis of macrocycles **4**–**6**.

boronate and vinyl bromide to afford ester **17C**. The hydrolysis of the ester moiety afforded acid **16C** in good yield.[6] The synthesis of amino acid **16B** required the preparation of organozinc species from iodide **18**. The intermediate *N*-Boc-protected organozinc species were cross-coupled with allyl bromide in the presence of stoichiometric amounts of copper bromide.[7] The formed aminohexenoate **17B** was converted into corresponding acid **16B** by saponification with LiOH (Scheme 1).[8]

Macrocycles **3**–**4** were prepared starting from oxazole **10** (Scheme 2). The allylamine moiety was installed in bromooxazole **10** or its *N*-deprotected derivative by S_NAr-type nucleophilic substitution of bromide in the presence of a general base such as NEt_3.[9] For the less

Scheme 1. Synthesis of oxazole building block **10** and amino acids **16B**,**C**

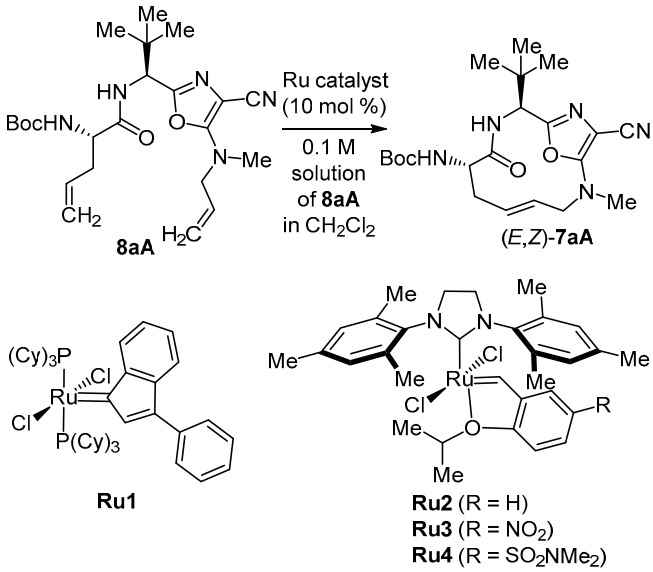


i: EDC·HCl, pyridine, rt, 24 h
ii: *t*-BuONO, $CuBr_2$, MeCN, rt, 1 h
iii: MeI, K_2CO_3, rt, DMF, 18 h
iv: 9-BBN, THF, 0°C to rt, 3 h, then aq 1 M Na_2CO_3, CH_2=CHBr, $Pd_2(dba)_3$ (2 mol %), P(*o*-tol)$_3$ (10 mol %), THF, –78°C to rt, 20 h
v: 1. Zn powder, I_2, DMF, rt, 3 h; 2. CuBr, CH_2=$CHCH_2Br$, –15°C to rt, DMF, 20 h
vi: LiOH, H_2O–1,4-dioxane, 1:1, rt, 3 h

nucleophilic aniline, the generation of the corresponding potassium anilide by KHMDS was required to effect the desired nucleophilic substitution of bromide in compound **10**. The cleavage of *N*-Cbz protection was accomplished by HBr solution, and higher yields were achieved if morpholine was added to trap the formed benzyl bromide and to avoid the alkylation side reactions. The amide bond formation with *N*-Boc-protected amino acids **16A**–**C** gave the corresponding dienes **8aA**–**C**,**bA**. *N*-Phenyl diene **8cA** was prepared by the initial amide coupling of intermediate **19** with amino acid **16A**, followed by regioselective allylation of the most nucleophilic nitrogen in the deprotonated compound **20** (Scheme 2).

Next, the reaction conditions for the RCM step were optimized using diene **8aA** as a model substrate (0.1 M solution in CH_2Cl_2) at 50°C[10] (Table 1). Initial attempts to effect the formation of the desired macrocycle **7aA** using catalyst **Ru1**[11] were not successful: incomplete conversion of the starting diene **8aA** was observed, and macrocycle **7aA** was not formed (entry 1). The lack of the RCM product and the incomplete conversion of diene **8aA** pointed to a low catalytic activity of **Ru1** that resulted in slow reaction and concomitant decomposition of the starting material under the tested conditions. Catalyst **Ru2** was also inefficient (entry 2),[12] however it was shown that the catalytic activity of complex **Ru2** could be increased by introduction of a strong electron-withdrawing group in the 2-isopropoxystyrene ligand.[13] Indeed, the nitro-substituted catalyst **Ru3** and the corresponding sulfonamide **Ru4** afforded 39 and 41%, respectively, of the desired macrocycle **7aA** together with ca. 20% of unidentified side products with the balance corresponding to 61–63% conversion (entries 3 and 4). We speculated that the metathesis-polymerization side reaction might account for the formation of the side products. Indeed, twofold increase of the dilution and prolonged reaction time has helped to increase the yield of macrocycle **7aA** to 63% isolated yield (entry 5). It should be noted that macrocycle **7aA** was formed as a 2:3 mixture of *E*:*Z* isomers that was anticipated for a medium-sized (12-membered) macrocycles.[14,15]

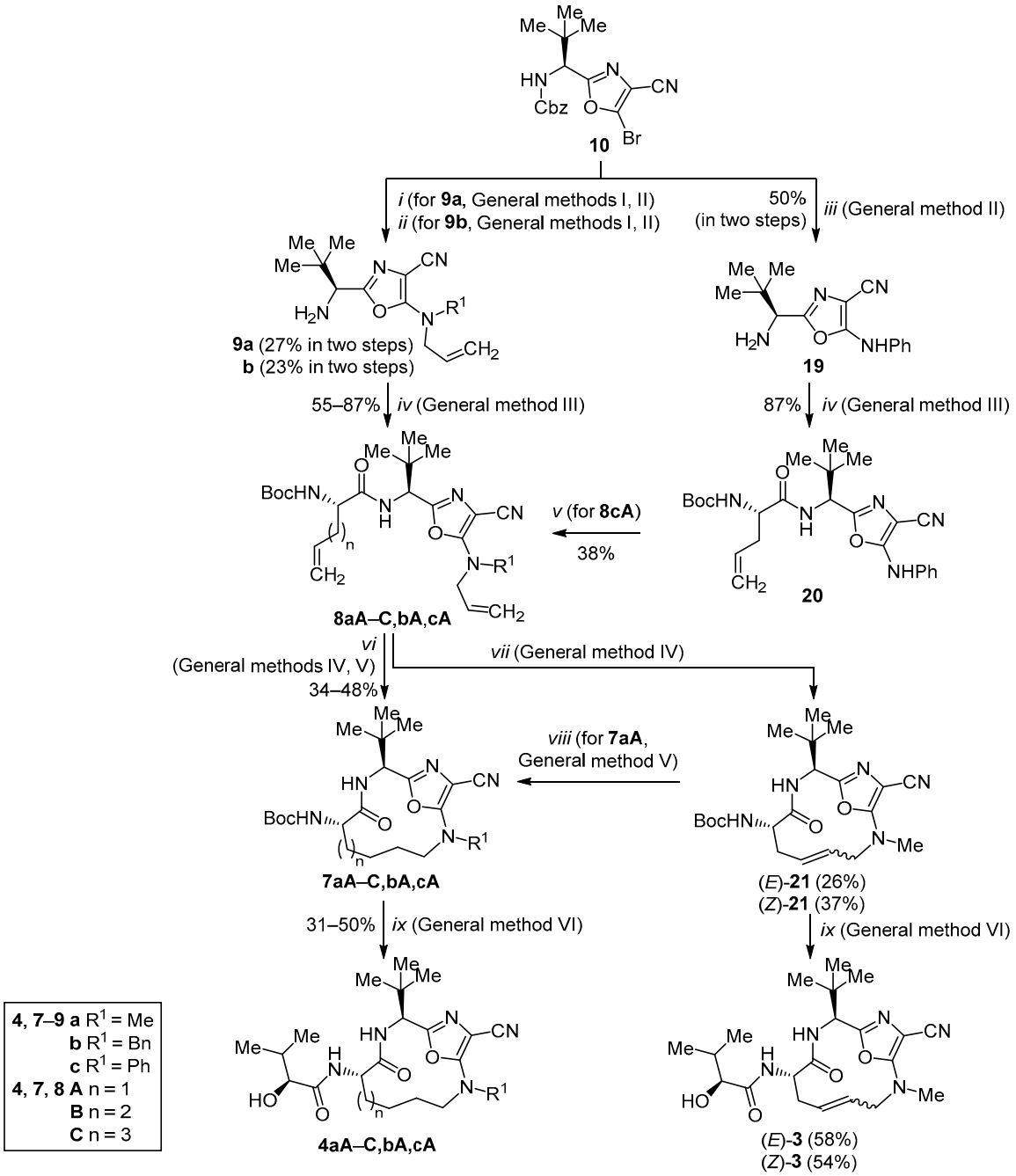
Table 1. Screening of conditions for the RCM*



Entry	Conditions	Conversion**, %	Yield**, %
1	**Ru1**, 50°C, 22 h	30	0
2	**Ru2**, 50°C, 22 h	100	0
3	**Ru3**, 50°C, 22 h	61	39
4	**Ru4**, 50°C, 22 h	63	41
5***	**Ru4**, 50°C, 72 h	83	76 (63*[4])

* 0.1 M solution of diene **8aA** in CH_2Cl_2 at 50°C.
** Determined by UPLC-MS assay.
*** 0.05 M solution.
*[4] Isolated yield.

Scheme 2. Synthesis of analogs **3** and **4aA**–**C**,**bA**,**cA**



i: 1. *N*-Methylallylamine (2 equiv), NEt_3 (3 equiv), DMF, rt, 18 h (General method I); 2. 33% HBr in AcOH (40 equiv), 1,4-dioxane, rt, 10 min, then morpholine, rt, 1 h (General method II)

ii: 1. 33% HBr in AcOH (40 equiv), 1,4-dioxane, rt, 10 min (General method II), then aqueous 2 M Na_2CO_3, rt; 2. *N*-allylbenzylamine (2 equiv), NEt_3 (3 equiv), DMF, rt, 18 h (General method I)

iii: 1. Aniline (2 equiv), $KN(SiMe_3)_2$ (3.5 equiv), THF, –78°C, 30 min; 2. 33% HBr in AcOH (40 equiv), 1,4-dioxane, rt, 10 min, then morpholine, rt, 1 h (General method II)

iv: Acid **16A**, **16B**, or **16C** (1.5 equiv), EDC·HCl (2 equiv), pyridine, rt, 18 h, 80% (**8aA**), 55% (**8aB**), 74% (**8aC**), 65% (**8bA**), 87% (**20**) (General method III)

v: NaH (3 equiv), allyl bromide (1 equiv), DMF, 0°C, 1 h

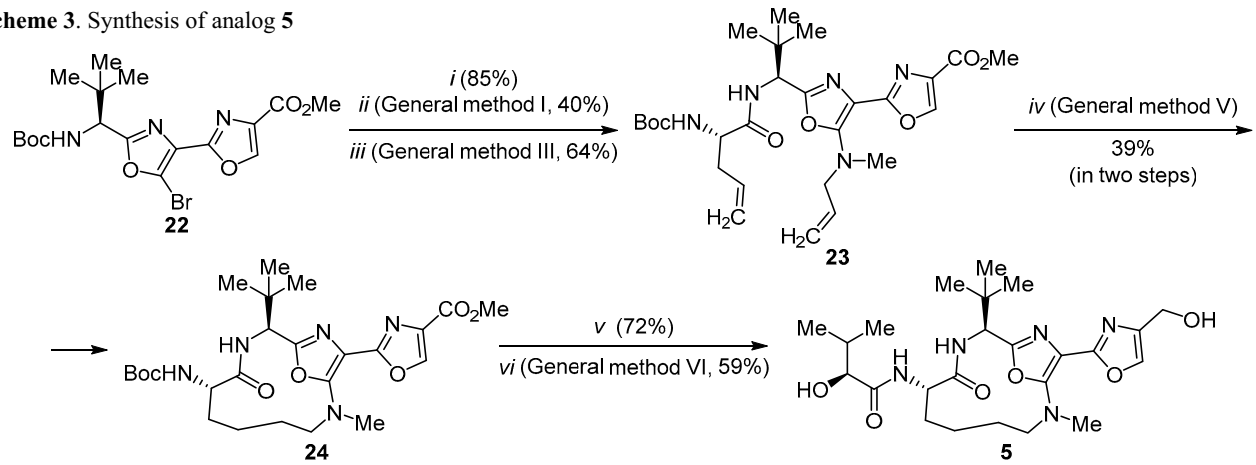
vi: 1. Catalyst **Ru4** (5 mol %), anhydrous CH_2Cl_2 (50 ml per mmol of alkene), rt, 30–48 h (General method IV); 2. H_2 (1 atm), 10% Pd/C (1 mol %), EtOAc, rt, 18–24 h (General method V); yield of the two-step process: 48% (**7aB**), 44% (**7aC**), 34% (**7bA**), 47% (**8cA**)

vii: 1. Catalyst **Ru4** (5 mol %), alkene **8aA**, CH_2Cl_2 (50 ml per mmol of alkene), rt, 30 h (General method IV); 2. Separation of *E*/*Z*-isomers by chromatography

viii: H_2 (1 atm), 10% Pd/C (1 mol %), EtOAc, rt, 18 h (General method V)

ix: 1. CF_3CO_2H (50 equiv), CH_2Cl_2, rt, 5 h; 2. (*S*)-2-hydroxy-3-methylbutanoic acid (1.5 equiv), EDC·HCl (3 equiv), HOBt (3 equiv), DIPEA (6 equiv), CH_2Cl_2, rt, 12 h (General method VI), 54% ((*Z*)-**3**), 58% ((*E*)-**3**), 50% (**4aA**), 46% (**4aB**), 49% (**4aC**), 31% (**4bA**), 50% (**4cA**)

Scheme 3. Synthesis of analog **5**



i: 33% HBr in AcOH (20 equiv), 1,4-dioxane, rt, 15 min
ii: *N*-Methylallylamine (1.6 equiv), NEt_3 (2 equiv), DMF, 50°C, 24 h (General method I)
iii: Acid **16A** (1.7 equiv), EDC·HCl (2 equiv), pyridine, rt, 3 h (General method III)
iv: 1. Catalyst **Ru4** (10 mol %), anhydrous CH_2Cl_2 (50 ml per mmol of alkene), 50°C, 24 h, then Ti(O*i*-Pr)$_4$ (1 equiv), 60°C, 5 days; 2. H_2 (1 atm), 10% Pd/C (1 mol %), EtOAc, rt, 20 h (General method V)
v: $LiBH_4$ (5 equiv), THF–CF_3CH_2OH, 10:1, rt, 20 h
vi: 1. CF_3CO_2H (50 equiv), CH_2Cl_2, rt, 3 h; 2. (*S*)-2-Hydroxy-3-methylbutanoic acid (1.5 equiv), EDC·HCl (3 equiv), HOBt (3 equiv), DIPEA (6 equiv), CH_2Cl_2, rt, 18 h (General method VI)

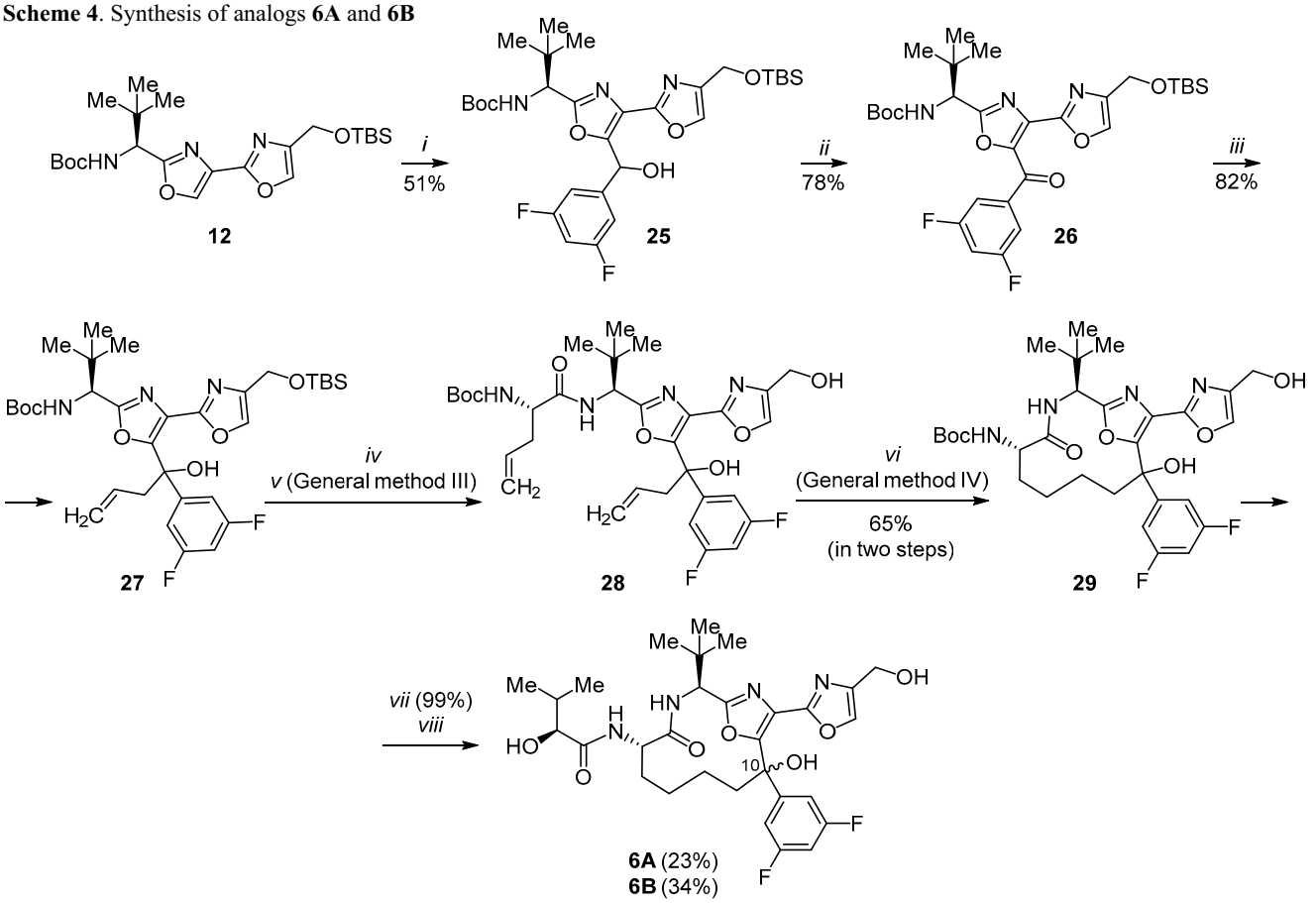
With the optimized cyclization conditions in hand, dienes **8aA**–**C**,**bA**,**cA** were subjected to RCM in the presence of catalyst **Ru4** (5 mol %) (Scheme 2). Isolation of the RCM products was accomplished only for diene **8aA** to afford unsaturated macrocycles (*E*)-**21** and (*Z*)-**21** as individual isomers after column chromatography. The other macrocyclization products were obtained as mixtures of *E*- and *Z*-isomers and directly converted into saturated macrocycles **7aA**–**C**,**bA**,**cA** under Pd-catalyzed hydrogenation conditions. The end-game of the synthesis involved *N*-Boc deprotection, followed by amide bond formation with (*S*)-2-hydroxy-3-methylbutanoic acid to afford unsaturated macrocycles (*E*)-**3** and (*Z*)-**3** as well as their saturated analogs **4aA**–**C**,**bA**,**cA** (Scheme 2).

Bioxazole-containing analog **5** was prepared by analogy to the synthesis of macrocycles **3**, **4** (Scheme 3). Thus, the *N*-Boc deprotection of compound **22**[4] was followed by the nucleophilic substitution of bromide with *N*-methylallylamine. Subsequent amide coupling with amino acid **16A** afforded diene **23**. Disappointingly, the optimized RCM conditions (Table 1, entry 5) turned out to be unsuitable for the RCM of diene **23**, as the formation of the desired macrocycle was not observed. We hypothesized that the chelation of catalytically active Ru species by substrate may be responsible for the inhibition of the catalytic cycle. It has been demonstrated that the addition of certain Lewis acids such as Ti(IV) species helps to avoid the deactivation of the Ru catalyst.[16] Indeed, the addition of stoichiometric amounts of Ti(O*i*-Pr)$_4$ improved the reaction rate and allowed for the formation of the unsaturated macrocycle, which was converted into the saturated analog **24** by Pd-catalyzed hydrogenation. The end-game of the synthesis involved reduction of the ester moiety, *N*-deprotection, and amide bond formation sequence to give macrocycle **5** (Scheme 3).

The synthesis of macrocycle **6** was started by regioselective lithiation of bioxazole **12**,[4] followed by the addition of the lithiated intermediate to 3,5-difluorobenzaldehyde to form alcohol **25** as a 1:1 mixture of diastereomers (Scheme 4). Subsequent oxidation with Dess–Martin periodinane[17] provided ketone **26**, which further reacted with allylmagnesium bromide to afford tertiary alcohol **27** (1:1 mixture of diastereomers). The cleavage of both *N*-Boc and OTBS protecting groups under acidic conditions was followed by the amide bond formation using acid **16A** to give diene **28**. The resulting diene was subjected to Ru-catalyzed RCM under the optimized conditions (Table 1, entry 5). The unsaturated macrocyclization product was formed as a 2:1 *E*:*Z* mixture of isomers in high (82%) yield. The mixture of isomers was hydrogenated to form macrocycle **29**. At this point, the stage was set for the *N*-deprotection/amide bond formation sequence. However, the cleavage of *N*-Boc protecting group in macrocycle **29** by trifluoroacetic acid resulted in the concomitant trifluoroacetoxylation of the tertiary alcohol-derived transient carbocation. After some experimentation, it was found that the ester side product can be hydrolyzed back to the tertiary alcohol by aqueous LiOH. Subsequent amide bond formation with (*S*)-2-hydroxy-3-methylbutanoic acid furnished macrocycle **6** as a mixture of diastereomers **6A** and **6B**. Individual diastereomers were obtained after separation by chromatography, however, the configuration of the quaternary stereogenic center could not be assigned for the individual diastereomers **6A**,**B** (Scheme 4).

The heat effect of binding (dissociation constants, K_d values) between all synthesized diazonamide A analogs **3**–**6** and α,β-tubulin tetramer (T4-RB3 complex) has been measured by isothermal titration calorimetry (ITC) method (Table 2). No heat effect of the binding has been observed for most of the synthesized analogs (Table 2, entries 1–4, 7, 8). Low micromolar binding was observed for diazonamide A analogs **4aC**,**bA** and both diastereomers of compound **6** (entries 5, 6, 9, and 10, respectively). However, the measured heat effects of binding are 2 orders

Scheme 4. Synthesis of analogs **6A** and **6B**



i: *n*-BuLi (2.1 equiv), Et_2O, –78°C, 10 min, then 3,5-difluorobenzaldehyde (2.4 equiv), –78°C to rt, 30 min
ii: Dess–Martin periodinane (1.5 equiv), CH_2Cl_2, rt, 1 h
iii: Allylmagnesium bromide (2.5 equiv), THF, –15°C, 30 min
iv: 4 M HCl in 1,4-dioxane, rt, 18 h, 69%
v: Acid **16A** (1.5 equiv), EDC·HCl (1.1 equiv), pyridine, rt, 3 h, 71% (General method III)
vi: 1. Catalyst **Ru4** (12.5 mol %), anhydrous CH_2Cl_2 (50 ml per mmol of alkene), rt, 44 h (General method IV);
2. H_2 (5 atm), 10% Pd/C (50 mol %), MeOH, rt, 5 days
vii: 1. CF_3CO_2H (65 equiv), CH_2Cl_2, rt, 3 h; 2. LiOH (5 equiv), THF–MeOH–H_2O, 1:1:1, rt, 1 h
viii: 1. (*S*)-2-Hydroxy-3-methylbutanoic acid (1.1 equiv), EDC·HCl (1.1 equiv), HOBt (1.1 equiv), DIPEA (3 equiv), CH_2Cl_2, rt, 18 h;
2. Separation of diastereomers by chromatography

of magnitude lower than that of DZ-2384 (**2**) (K_d 0.05 μM). Hence, the ITC results provided strong evidence that the presence of the tetracyclic subunit GHFE in the natural product **1** and the synthetic derivative **2** is important for the binding of these antimitotic agents to the α,β-tubulin tetramer.

Table 2. Heat effect of binding between compounds **3**–**6** and α,β-tubulin tetramer (T4-RB3 complex) measured by ITC method

Entry	Compound	K_d, μM*
1	(*E*)-**3**	No heat observed
2	(*Z*)-**3**	No heat observed
3	**4aA**	No heat observed
4	**4aB**	No heat observed
5	**4aC**	2.52 ± 0.83**
6	**4bA**	3.38
7	**4cA**	No heat observed
8	**5**	No heat observed
9	**6A**	4.10
10	**6B**	1.52

* Single ITC experiment.
** Duplicate ITC run.

A series of structurally simplified macrocyclic analogs of marine metabolite diazonamide A and synthetic antimitotic agent DZ-2384 have been synthesized in 7 to 9 linear steps from chiral, enantiomerically pure 5-bromooxazole building block. Ruthenium-catalyzed ring-closing metathesis was employed to construct 12–14-membered macrocycles. The addition of stoichiometric amounts of Ti(*i*-OPr)$_4$ has helped to avoid the chelation of catalytically active Ru species by bioxazole subunit-containing RCM substrates. The starting chiral 5-bromooxazole building block was prepared from (*S*)-*tert*-leucine in a straightforward two-step synthesis. Several of the synthesized diazonamide analogs showed weak (low micromolar) binding with α,β-tubulin tetramer (T4-RB3 complex), and the measured heat effects were considerably lower than that of DZ-2384. These data provided an evidence that the presence of the tetracyclic subunit GHFE in diazonamide A and its synthetic derivative DZ-2384 is important for the

binding to the α,β-tubulin tetramer. We believe that this finding will have implications in the design of diazonamide A analogs as antimitotic agents.

Experimental

^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance Neo spectrometer with a 5-mm Double-Resonance Broadband CryoProbe Prodigy probe (400 and 101 MHz, respectively) using TMS or the residual solvent peaks as internal reference ($CDCl_3$: 7.26 ppm for ^{1}H nuclei and 77.2 ppm for ^{13}C nuclei; DMSO-d_6: 2.50 ppm for ^{1}H nuclei and 39.5 ppm or ^{13}C nuclei; CD_3OD: 3.31 ppm for ^{1}H nuclei and 49.0 ppm for ^{13}C nuclei). Assignments in ^{13}C NMR spectra of compounds **20**, (*Z*)-**3**, **4bA** and **6B** were made based on the COSY, ^{1}H–^{13}C HSQC, and ^{1}H–^{13}C HMBC experiments. High-resolution mass spectra were recorded on a Waters Synapt G2-Si TOF MS instrument using ESI technique. Specific rotation was recorded on a Kruss P3000 instrument. Analytical thin-layer chromatography (TLC) was performed on precoated silica gel F-254 plates (Merck).

All chemicals were used as obtained from commercial sources and all reactions were performed under argon atmosphere in an oven-dried (120°C) glassware, unless noted otherwise. Anhydrous PhMe, Et_2O, THF, and CH_2Cl_2 were obtained by passing commercially available solvent through activated alumina columns.

General method I. Allylamine (2 equiv), followed by NEt_3 (3 equiv), was dropwise added to a solution of bromooxazole or bromobioxazole (1 equiv) in DMF (6 ml/mmol) at room temperature. The orange solution was stirred for 18–24 h at room temperature or at 50°C. Then the reaction mixture was diluted with EtOAc (10 ml/mmol) and washed with H_2O (10 ml/mmol) two times. The organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel using gradient elution from pure hexane to 30% EtOAc in hexane or from pure CH_2Cl_2 to 10% MeOH in CH_2Cl_2.

General method II. 33% HBr solution in AcOH (60 equiv) was added dropwise to a solution of Cbz-protected aminooxazole (1 equiv) in 1,4-dioxane (3 ml/mmol) at room temperature. The yellow solution was stirred for 10 min, then cooled to 0°C (crushed ice batch), and 2 M aqueous Na_2CO_3 solution was carefully added (*Caution! Intense gas evolution!*) until the mixture medium reached pH 7–9. The mixture was extracted with EtOAc (10 ml/mmol) four times. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and filtered. Morpholine (neat, 10 equiv) was added, and after stirring for 1 h at room temperature, all volatiles were removed under reduced pressure.

General method III. *N*-Boc-protected carboxylic acid **16** (1.5 equiv) was added at room temperature to a solution of amine **9a**,**b**, **19**, **22**, **27** (1 equiv) and EDC·HCl (2 equiv) in anhydrous pyridine (15 ml/mmol). The yellow solution was stirred for 3–18 h at room temperature, then pyridine was evaporated under reduced pressure. The residue was dissolved in EtOAc (10 ml/mmol) and washed with 0.1 M aqueous HCl solution two times. The organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel using gradient elution from pure hexane to 40% EtOAc in hexane.

General method IV. Catalyst **Ru4** (0.05 equiv) and diene **8aA**–**C**,**bA**,**cA**, **28** (1 equiv) were weighed in an oven-dried ACE glass pressure tube, and anhydrous CH_2Cl_2 (50 ml/mmol) was added. The resulting green solution was stirred at 60°C for 30–48 h whereupon the color gradually changed to dark-brown. The reaction mixture was cooled to room temperature and concentrated under reduced pressure.

General method V. 10% Pd on carbon (0.01 equiv) was added to a solution of macrocyclic alkene (from General method III or IV) in EtOAc (15 ml/mmol) under argon atmosphere. The argon atmosphere was replaced by hydrogen atmosphere and the black suspension was stirred for 18–24 h at room temperature. Then the suspension was filtered through a pad of Celite®, and the filtrate was concentrated under reduced pressure. The residue was purified by reversed-phase column chromatography on silica gel using gradient elution from pure H_2O to 95% MeCN in H_2O.

General method VI. CF_3CO_2H (50 equiv) was dropwise added to a solution of macrocycle **7aA–C**,**bA**,**cA**, (*Z*)-**21**, or (*E*)-**21** (1 equiv) in CH_2Cl_2 (10 ml/mmol) at 0°C. The resulting solution was warmed to room temperature and stirred for 3–5 h, whereupon all volatiles were concentrated under reduced pressure. The resulting crude amine was used in the next step without further purification. Thus, (*S*)-2-hydroxy-3-methylbutanoic acid (1.5 equiv), EDC·HCl (3 equiv), and HOBt (3 equiv) were added to a solution of the above crude amine in CH_2Cl_2 (15 ml/mmol). Then DIPEA (6 equiv) was added dropwise and the resulting yellow solution was stirred at room temperature for 12–18 h, whereupon the mixture was washed with 1 M aqueous HCl (10 ml/mmol) two times. The organic layers were washed with brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was purified by reversed-phase column chromatography on silica gel using gradient elution from pure H_2O to 95% MeCN in H_2O.

Benzyl *N*-[(1*S*)-1-(5-amino-4-cyano-1,3-oxazol-2-yl)-2,2-dimethylpropyl]carbamate (15). EDC·HCl (16.3 g, 85.2 mmol, 1.2 equiv) was added to a solution of (2*S*)-2-{([benzyloxy)carbonyl]amino}-3,3-dimethylbutanoic acid (**13**)[5,18] (18.8 g, 71.0 mmol, 1 equiv) and aminomalononitrile *p*-toluenesulfonate (**14**) (19.8 g, 78.1 mmol, 1.1 equiv) in pyridine (200 ml). The resulting orange solution was stirred for 24 h at room temperature, whereupon pyridine was evaporated under reduced pressure. The residue was dissolved in EtOAc and washed with H_2O three times. The organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure. The crude product was used in the next reaction without additional purification. Yield 21.5 g (92%), brown amorphous solid, $[\alpha]_D^{20}$ –41.4° (*c* 1.0, MeOH). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 7.38–7.32 (5H, m, H Ph); 5.34 (1H, d, *J* = 9.8, NH); 5.12–5.09 (2H, m, CH_2); 4.81 (2H, s, NH_2); 4.58 (1H, d, *J* = 9.8, CH); 0.98 (9H, s, $C(CH_3)_3$). ^{13}C NMR spectrum (DMSO-d_6), δ, ppm: 162.3; 156.7; 151.9; 137.3; 128.8; 128.7; 128.2; 115.9; 82.8; 66.1; 58.2; 35.0; 26.7. Found, *m*/*z*: 329.1614 $[M+H]^+$. $C_{17}H_{21}N_4O_3$. Calculated, *m*/*z*: 329.1614.

Benzyl *N*-[(1*S*)-1-(5-bromo-4-cyano-1,3-oxazol-2-yl)-2,2-dimethylpropyl]carbamate (10). *tert*-Butylnitrite (1.2 ml, 10.1 mmol, 1.1 equiv) was added to a suspension of $CuBr_2$ (4.1 g, 18.3 mmol, 2 equiv) in MeCN (80 ml) at room temperature under argon atmosphere. The resulting solution was stirred for 5 min whereupon a solution of amine **15** (3.0 g, 9.1 mmol, 1 equiv) in MeCN (30 ml) was added dropwise at room temperature. *Gas evolution was observed!* The reaction mixture was stirred for 1 h at room temperature. Then Et_2O and H_2O were added and layers were separated. The organic layer was washed with 1 M aqueous HCl solution (3×20 ml), then with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by reversed-phase column chromatography on silica gel using gradient elution from 40 to 85% MeCN in H_2O to give product as a brown oil, which was additionally purified by direct-phase column chromatography on silica gel using gradient elution from pure hexane to 30% EtOAc in hexane. Yield 1.6 g (46%), colorless oil, $[\alpha]_D^{20}$ –17.7° (*c* 1.0, $CHCl_3$). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 7.40–7.29 (5H, m, H Ph); 5.40 (1H, d, *J* = 9.7, NH); 5.10 (2H, s, CH_2); 4.76 (1H, d, *J* = 9.7, CH); 1.00 (9H, s, $C(CH_3)_3$). ^{13}C NMR ($CDCl_3$), δ, ppm: 166.0; 155.9; 135.8; 130.9; 128.6; 128.4; 128.2; 116.0; 110.8; 67.5; 58.1; 35.5; 26.1. Found, *m*/*z*: 392.0603 $[M+H]^+$. $C_{17}H_{19}BrN_3O_3$. Calculated, *m*/*z*: 392.0610.

Methyl (2*S*)-2-[(*tert*-butoxycarbonyl)amino]hex-5-enoate (17B). An oven-dried Schlenk flask (50 ml) was charged with Zn powder (2.2 g, 34.0 mmol, 4 equiv) and attached to high vacuum system (0.1 Torr). The flask was heated with heatgun and then cooled back to room temperature. The heating/cooling sequence was repeated three times. Then the flask was disconnected from the vacuum, filled with argon, and I_2 (533 mg, 2.1 mmol, 0.3 equiv) was added. The flask was heated again with the heatgun until evaporation of I_2 has started (red steam appeared). The flask was cooled to room temperature under argon atmosphere, and anhydrous DMF (10 ml) was added dropwise, whereupon color of the suspension slowly (within 1 min) changed from dark-brown to colorless. The resulting suspension was cooled to 0°C (crushed ice), and a solution of methyl (2*R*)-2-[(*tert*-butoxycarbonyl)amino]-3-iodopropanoate (**18**) (2.3 g, 7.0 mmol, 1 equiv) in anhydrous DMF (7 ml) was added dropwise. The resulting yellow suspension was warmed to room temperature and stirred for 3 h, whereupon the stirring was stopped to let the solid settle to the bottom. The supernatant containing organozinc species (~0.5 M, 8.4 ml, 4.2 mmol, 1 equiv) was then carefully transferred by a syringe to a well-stirred suspension of CuBr (301 mg, 2.1 mmol, 0.5 equiv) and allyl bromide (0.7 ml, 8.4 mmol, 2 equiv) in anhydrous DMF (5 ml) at –15°C (NaCl–ice bath). After the addition was completed, the cooling bath was removed and the stirring was continued for 20 h. Then, EtOAc (25 ml) was added to the reaction mixture and stirring was continued for 15 min. The mixture was washed with H_2O (20 ml), the organic layer was washed with aqueous 1 M $Na_2S_2O_3$ (2×20 ml), then with H_2O (20 ml) and brine. After drying over anhydrous Na_2SO_4 and concentration under reduced pressure, the residue was purified by column chromatography on silica gel using gradient elution from pure hexane to 10% EtOAc in hexane. Yield 716 mg (70%), colorless oil. ^{1}H NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 5.79 (1H, ddt, *J* = 16.9, *J* = 10.2, *J* = 6.5, CH_2=C<u>H</u>); 5.09–5.04 (1H, m, CH); 5.04–4.95 (2H, m, =CH_2); 4.30–4.28 (1H, m, NH); 3.74 (3H, s, OCH_3); 2.19–2.00 (2H, m, CH_2); 1.95–1.89 (1H, m, CH_2); 1.78–1.56 (1H, m, CH_2); 1.63 (9H, s, $C(CH_3)_3$). NMR data are in agreement with the literature reported.[7]

Methyl (2*S*)-2-[(*tert*-butoxycarbonyl)amino]hept-6-enoate (17C). Anhydrous K_2CO_3 (2.7 g, 19.5 mmol, 3 equiv) and MeI (1.2 ml, 19.5 mmol, 3 equiv) were added to a solution of (2*S*)-2-[(*tert*-butoxycarbonyl)amino]pent-4-enoic acid (**16A**) (1.4 g, 6.5 mmol, 1 equiv) in anhydrous DMF (30 ml) at room temperature. The yellow suspension was stirred for 18 h, whereupon it was diluted with Et_2O (50 ml), washed with H_2O (2×50 ml), then with aqueous 10% $CuSO_4$ solution (50 ml) and brine. The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give methyl ester **17A** as a yellow oil, yield 1.4 g (96%), which was used in the next step without additional purification.

A solution of 9-BBN in THF (0.5 M, 26 ml, 13.1 mmol, 2.5 equiv) was added to a solution of methyl ester **17A** (1.2 g, 5.2 mmol, 1 equiv) in anhydrous THF (20 ml) at 0°C (crushed ice). The mixture was warmed to room temperature, stirred for 3 h, and then quenched with aqueous 1 M Na_2CO_3 solution (18 ml, 18.3 mmol, 3.5 equiv) while passing a stream of argon through the resulting solution. Vinyl bromide (1 M solution in THF, 21 ml, 20.9 mmol, 4 equiv), followed by a degassed solution of boronate from above was added to a stirred suspension of $Pd_2(dba)_3$ (96 mg, 0.1 mmol, 0.02 equiv) and tri-*o*-tolylphosphine (159 mg, 0.5 mmol, 0.1 equiv) in THF at −78°C (dry ice/Me_2CO bath). The resulting mixture was warmed to room temperature and stirred for 20 h, whereupon the dark-brown solution was diluted with H_2O (50 ml) and extracted with EtOAc (3×50 ml). The organic extracts were combined, washed with brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel using gradient elution from pure hexanes to 10% EtOAc in hexane. Yield 1.1 g (81%), colorless oil. ^{1}H NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 5.83–5.68 (1H, m, C<u>H</u>=CH_2); 5.05–4.91 (3H, m, C<u>H</u>$_2$=CH, CH); 4.30–4.28 (1H, m, NH); 3.73 (3H, s, OCH_3); 2.11–2.01 (2H, m, CH_2); 1.90–1.75 (1H, m, CH_2); 1.70–1.60 (1H, m, CH_2); 1.55–1.38 (11H, m, $C(CH_3)_3$, CH_2). NMR data are in agreement with the literature reported.[6]

(2*S*)-2-[(*tert*-Butoxycarbonyl)amino]hex-5-enoic acid (16B). A solution of LiOH (44 mg, 1.9 mmol, 1 equiv) in H_2O (10 ml) was added to a solution of ester **17B** (450 mg, 1.9 mmol, 1 equiv) in 1,4-dioxane (10 ml). The colorless mixture was stirred at room temperature for 3 h and concentrated under reduced pressure. The residue was partitioned between H_2O (20 ml) and EtOAc (20 ml). The aqueous phase was acidified with 1 M HCl to pH 4 and extracted with EtOAc (2×20 ml). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. Yield 424 mg (99%), colorless oil. ^{1}H NMR ($CDCl_3$), δ, ppm (*J*, Hz): 7.89 (1H, br. s, OH); 5.76–5.68 (1H, m, C<u>H</u>=CH_2); 5.03–4.89 (3H, m, C<u>H</u>$_2$=CH, CH); 4.27–4.23 (1H, m, NH);

2.12–2.03 (2H, m, CH_2); 1.92–1.84 (1H, m, CH_2); 1.74–1.68 (1H, m, CH_2); 1.39 (9H, s, $C(CH_3)_3$). NMR data are in agreement with the literature reported.[8]

(2*S*)-2-[(*tert*-Butoxycarbonyl)amino]hept-6-enoic acid (16C). A solution of LiOH (101 mg, 4.2 mmol, 1 equiv) in H_2O (25 ml) was added to a solution of ester **17C** (1.08 g, 4.2 mmol, 1 equiv) in 1,4-dioxane (25 ml). The colorless mixture was stirred at room temperature for 3 h and evaporated under reduced pressure. The residue was partitioned between H_2O (50 ml) and EtOAc (50 ml). The aqueous phase was acidified with 1 M HCl to pH 4 and extracted with EtOAc (2×20 ml). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. Yield 922 mg (90%), colorless oil. ^{1}H NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 9.53 (1H, br. s, OH); 5.85–5.69 (1H, m, C<u>H</u>=CH_2); 5.06–4.93 (3H, m, C<u>H</u>$_2$=CH, CH); 4.34–4.30 (1H, m, NH); 2.13–2.03 (2H, m, CH_2); 1.95–1.76 (1H, m, CH_2); 1.73–1.59 (1H, m, CH_2); 1.56–1.37 (11H, m, $C(CH_3)_3$, CH_2). NMR data are in agreement with the literature reported.[6]

2-((1*S*)-1-Amino-2,2-dimethylpropyl)-5-[methyl(prop-2-en-1-yl)amino]-1,3-oxazole-4-carbonitrile (**9a**) was obtained in a two-step sequence from benzyl *N*-[(1*S*)-1-(5-bromo-4-cyano-1,3-oxazol-2-yl)-2,2-dimethylpropyl]carbamate (**10**).

Step 1. **Benzyl *N*-[(1*S*)-1-{4-cyano-5-[methyl(prop-2-en-1-yl)amino]-1,3-oxazol-2-yl}-2,2-dimethylpropyl]carbamate** was obtained from bromooxazole **10** (6.0 g, 15.2 mmol), *N*-methylallylamine (2.8 ml, 30.8 mmol), and NEt_3 (6.4 ml, 46.0 mmol) in anhydrous DMF following the general method I. The crude product was purified by column chromatography on silica gel using gradient elution from pure hexanes to 30% EtOAc in hexane to afford the subtitle compound. Yield 4.80 g (82%), yellow oil. $[\alpha]_D^{20}$ –30.6° (*c* 1.0, $CHCl_3$). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 7.39–7.27 (5H, m, H Ph); 5.86–5.73 (1H, m, C<u>H</u>=CH_2); 5.43 (1H, d, *J* = 9.8, CH(NH)); 5.31–5.20 (2H, m, CH_2=); 5.10 (2H, s, CH_2O); 4.57 (1H, d, *J* = 9.8, NH(CH)); 3.97 (2H, dq, *J* = 14.0, *J* = 5.7, NCH_2); 3.10 (3H, s, CH_3); 0.97 (9H, s, $C(CH_3)_3$). ^{13}C NMR spectrum ($CDCl_3$), δ, ppm: 160.0; 156.1; 152.1; 136.1; 131.1; 128.5; 128.2; 128.1; 118.9; 116.0; 84.5; 67.2; 57.4; 53.9; 36.3; 35.7; 26.1. Found, *m*/*z*: 383.2083 $[M+H]^+$. $C_{21}H_{27}N_4O_3$. Calculated, *m*/*z*: 383.2092.

Step 2. **2-((1*S*)-1-Amino-2,2-dimethylpropyl)-5-[methyl-(prop-2-en-1-yl)amino]-1,3-oxazole-4-carbonitrile** (**9a**) was obtained from benzyl *N*-[(1*S*)-1-{4-cyano-5-[methyl-(prop-2-en-1-yl)amino]-1,3-oxazol-2-yl}-2,2-dimethylpropyl]-carbamate (step 1) (3.57 g, 9.3 mmol) and 33% HBr in AcOH (96 ml, 560.1 mmol) in 1,4-dioxane, followed by the addition of morpholine (8.1 ml, 93.3 mmol) by the general method II. Yield 762 mg (33%), orange oil. The crude amine **9a** was used in the next step without additional purification.

***tert*-Butyl *N*-{(1*S*)-1-[((1*S*)-1-{4-cyano-5-[methyl(prop-2-en-1-yl)amino]-1,3-oxazol-2-yl}-2,2-dimethylpropyl)-carbamoyl]but-3-en-1-yl}carbamate (8aA)** was obtained from crude amine **9a** (254 mg, 1.0 mmol), EDC·HCl (292 mg, 2.0 mmol), and acid **16A** (330 mg, 1.5 mmol) in anhydrous pyridine following the general method III. Yield 365 mg (80%), colorless oil, $[\alpha]_D^{20}$ –33.5° (*c* 1.3, $CDCl_3$). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 6.91 (1H, br. s, NH); 5.86–5.68 (2H, m, 2C<u>H</u>=CH_2); 5.31–5.20 (2H, m, CH=C<u>H</u>$_2$); 5.19–5.10 (2H, m, CH=C<u>H</u>$_2$); 4.95 (1H, br. s, NH); 4.84 (1H, d, *J* = 9.4, CH–oxazole); 4.16–4.08 (1H, m, C<u>H</u>CH_2); 4.05–3.90 (2H, m, NCH_2); 3.11 (3H, s, CH_3); 2.57–2.43 (2H, m, CHC<u>H</u>$_2$); 1.44 (9H, s, $C(CH_3)_3$); 0.95 (9H, s, $C(CH_3)_3$). ^{13}C NMR spectrum ($CDCl_3$), δ, ppm: 171.1; 160.0; 155.7; 151.7; 133.0; 131.1; 119.2; 118.9; 116.0; 84.5; 80.6; 55.0; 53.9; 36.3; 35.8; 28.3; 26.1. Found, *m*/*z*: 446.2771 $[M+H]^+$. $C_{23}H_{36}N_5O_4$. Calculated, *m*/*z*: 446.2767.

***tert*-Butyl *N*-{(1*S*)-1-[((1*S*)-1-{4-cyano-5-[methyl(prop-2-en-1-yl)amino]-1,3-oxazol-2-yl}-2,2-dimethylpropyl)-carbamoyl]pent-4-en-1-yl}carbamate (8aB)** was obtained from crude amine **9a** (250 mg, 1.0 mmol), acid **16B** (231 mg, 1.0 mmol), and EDC·HCl (232 mg, 1.2 mmol) in anhydrous pyridine following the general method III. Yield 253 mg (55%), yellow oil, $[\alpha]_D^{20}$ –42.5° (*c* 1.0, $CHCl_3$). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 6.80 (1H, d, *J* = 9.0, CHN<u>H</u>); 5.87–5.69 (2H, m, 2C<u>H</u>=CH_2); 5.32–5.18 (2H, m, CH=C<u>H</u>$_2$); 5.09–4.97 (2H, m, CH=C<u>H</u>$_2$); 4.95 (1H, d, *J* = 7.4, N<u>H</u>COO); 4.84 (1H, d, *J* = 9.0, C<u>H</u>–oxazole); 4.07–3.89 (3H, m, C<u>H</u>$_2$–CH=, CHCO); 3.11 (3H, s, CH_3); 2.17–2.06 (2H, m, CH_2); 1.99–1.85 (1H, m, CH_2); 1.76–1.63 (1H, m, CH_2); 1.44 (9H, s, $C(CH_3)_3$); 0.96 (9H, s, $C(CH_3)_3$). ^{13}C NMR spectrum ($CDCl_3$), δ, ppm: 171.6; 160.1; 155.7; 151.7; 137.1; 131.1; 118.9; 116.0; 115.9; 84.5; 80.4; 55.0; 54.2; 53.9; 36.4; 35.8; 30.7; 29.8; 28.3; 26.1. Found, *m*/*z*: 460.2925 $[M+H]^+$. $C_{24}H_{38}N_5O_4$. Calculated, *m*/*z*: 460.2924.

***tert*-Butyl *N*-{(1*S*)-1-[((1*S*)-1-{4-cyano-5-[methyl(prop-2-en-1-yl)amino]-1,3-oxazol-2-yl}-2,2-dimethylpropyl)-carbamoyl]hex-5-en-1-yl}carbamate (8aC)** was obtained from crude amine **9a** (350 mg, 1.4 mmol), acid **16C** (343 mg, 1.4 mmol), and EDC·HCl (324 mg, 1.7 mmol) in anhydrous pyridine following the general method III. Yield 495 mg (74%), yellow oil, $[\alpha]_D^{20}$ –42.3° (*c* 1.0, $CHCl_3$). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 6.78 (1H, d, *J* = 8.8, CHN<u>H</u>); 5.88–5.68 (2H, m, 2C<u>H</u>=CH_2); 5.31–5.20 (2H, m, CH=C<u>H</u>$_2$); 5.05–4.92 (2H, m, CH=C<u>H</u>$_2$); 4.91–4.87 (1H, m, N<u>H</u>COO); 4.84 (1H, d, *J* = 9.5, C<u>H</u>–oxazole); 4.07–3.88 (3H, m, C<u>H</u>$_2$–CH=, CHCO); 3.11 (3H, s, CH_3); 2.11–2.00 (2H, m, CH_2); 1.87–1.81 (1H, m, CH_2); 1.71–1.55 (1H, m, CH_2); 1.49–1.42 (11H, m, $C(CH_3)_3$,CH_2); 0.98 (9H, s, $C(CH_3)_3$). ^{13}C NMR spectrum ($CDCl_3$), δ, ppm: 171.8; 160.2; 151.9; 138.1; 131.3; 119.1; 116.1; 115.3; 84.7; 54.8; 55.1; 54.1; 36.5; 36.0; 33.4; 32.2; 31.2; 28.5; 26.4; 26.3; 25.1. Found, *m*/*z*: 474.3086 $[M+H]^+$. $C_{25}H_{40}N_5O_4$. Calculated, *m*/*z*: 474.3080.

2-((1*S*)-1-Amino-2,2-dimethylpropyl)-5-[benzyl(prop-2-en-1-yl)amino]-1,3-oxazole-4-carbonitrile (9b) was obtained in a two-step sequence from benzyl *N*-[(1*S*)-1-(5-bromo-4-cyano-1,3-oxazol-2-yl)-2,2-dimethylpropyl]carbamate (**10**).

Step 1. **2-((1*S*)-1-Amino-2,2-dimethylpropyl)-5-bromo-1,3-oxazole-4-carbonitrile**. 33% HBr solution in AcOH (28 ml, 163 mmol, 40 equiv) was added dropwise to a solution of bromooxazole **10** (1.6 g, 4.1 mmol, 1 equiv) in 1,4-dioxane (15 ml) at room temperature. The yellow solution was stirred for 10 min, and cooled (0°C) 2 M Na_2CO_3 aqueous solution was added till the mixture medium reached pH 7–9. The mixture was extracted with EtOAc (4×50 ml). The organic layers were combined and washed with 1 M HCl aqueous solution (2×50 ml). Aqueous layers were combined, and solid Na_2CO_3 was added till the medium reached pH 9. Aqueous layer was

extracted with EtOAc (3×50 ml). The organic layers were combined, washed with brine, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. The crude subtitle product was obtained as orange oil, yield 700 mg (67%), and used in the next reaction without additional purification.

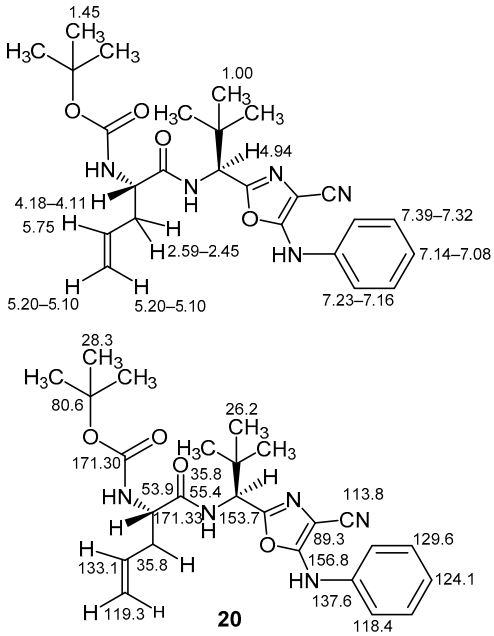
Step 2. **2-((1*S*)-1-Amino-2,2-dimethylpropyl)-5-[benzyl-(prop-2-en-1-yl)amino]-1,3-oxazole-4-carbonitrile (9b)** was obtained from 2-[(1*S*)-1-amino-2,2-dimethylpropyl]-5-bromo-1,3-oxazole-4-carbonitrile (700 mg, 2.7 mmol), *N*-allylbenzylamine (0.8 ml, 5.4 mmol), and NEt_3 (1.1 ml, 8.1 mmol) in anhydrous DMF 50°C (72 h) following the general method I. Purification by column chromatography on silica gel using gradient elution from pure hexane to 30% EtOAc in hexane afforded compound **9b**. Yield 295 mg (34%), yellow oil. Compound **9b** was used in the next reaction without additional purification.

***tert*-Butyl *N*-{(1*S*)-1-[((1*S*)-1-{5-[benzyl(prop-2-en-1-yl)amino]-4-cyano-1,3-oxazol-2-yl}-2,2-dimethylpropyl)-carbamoyl]but-3-en-1-yl}carbamate (8bA)** was obtained from compound **9b** (295 mg, 0.9 mmol), acid **16A** (196 mg, 0.9 mmol), and EDC·HCl (209 mg, 1.1 mmol) in anhydrous pyridine following the general method III. Yield 310 mg (65%), yellow oil, $[\alpha]_D^{20}$ –28.0° (*c* 1.0, $CHCl_3$). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 7.40–7.20 (5H, m, H Ph); 6.97–6.86 (1H, m, NH); 5.88–5.66 (2H, m, 2C<u>H</u>=CH_2); 5.32–5.23 (2H, m, CH=C<u>H</u>$_2$); 5.21–5.09 (2H, m, CH=C<u>H</u>$_2$); 4.98–4.89 (1H, m, NH); 4.84 (1H, d, *J* = 9.5, C<u>H</u>–oxazole); 4.69–4.48 (2H, m, C<u>H</u>$_2$Ph); 4.17–4.06 (1H, m, CHCO); 4.05–3.87 (2H, m, C<u>H</u>$_2$–CH=); 2.59–2.40 (2H, m, C<u>H</u>$_2$–CH=); 1.44 (9H, s, $C(CH_3)_3$); 0.92 (9H, s, $C(CH_3)_3$). ^{13}C NMR spectrum ($CDCl_3$), δ, ppm: 171.1; 159.9; 155.7; 151.8; 135.5; 133.0; 131.2; 128.9; 128.1; 127.7; 119.2; 155.7; 84.8; 80.6; 55.0; 53.9; 52.4; 51.1; 35.9; 29.7; 28.3; 26.1. Found, *m*/*z*: 522.3083 $[M+H]^+$. $C_{29}H_{40}N_5O_4$. Calculated, *m*/*z*: 522.3080.

2-((1*S*)-1-Amino-2,2-dimethylpropyl)-5-(phenylamino)-1,3-oxazole-4-carbonitrile (19) was obtained in a two-step sequence from benzyl *N*-[(1*S*)-1-(5-bromo-4-cyano-1,3-oxazol-2-yl)-2,2-dimethylpropyl]carbamate (**10**).

Step 1. **Benzyl *N*-{(1*S*)-1-[4-cyano-5-(phenylamino)-1,3-oxazol-2-yl]-2,2-dimethylpropyl}carbamate**. Aniline (0.4 ml, 4.2 mmol, 2 equiv) was added dropwise to a solution of bromooxazole **10** (815 mg, 2.1 mmol, 1 equiv) in anhydrous THF (20 ml) at room temperature. Then the orange solution was cooled to –78°C (dry ice/Me_2CO) and KHMDS (1 M solution in THF (7.3 ml, 7.3 mmol, 3.5 equiv)) was added dropwise. The resulting dark solution was stirred for 30 min at –78°C and quenched by the addition of aqueous saturated NH_4Cl solution. The organic layer was diluted with EtOAc (50 ml), washed with aqueous 1 M HCl (2×40 ml), brine, and dried over anhydrous Na_2SO_4. Filtration and evaporation under reduced pressure afforded the subtitle compound as a yellow amorphous solid, yield 760 mg (90%), $[\alpha]_D^{20}$ –7.6° (*c* 1.0, $CHCl_3$). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 7.50 (1H, br. s, N<u>H</u>Ph); 7.40–7.27 (7H, m, H Ph); 7.22–7.08 (3H, m, H Ph); 5.45 (1H, d, *J* = 9.8, CH(NH)); 5.14–5.09 (2H, m, CH_2); 4.69 (1H, d, *J* = 9.8, NH(CH)); 1.02 (9H, s, $C(CH_3)_3$). ^{13}C NMR spectrum ($CDCl_3$), δ, ppm: 156.9; 156.1; 154.0; 137.6; 136.0; 129.6; 128.6; 128.3; 128.2; 124.1; 118.3; 113.9; 89.2; 67.4; 57.8; 35.7; 26.2. Found, *m*/*z*: 405.1928 $[M+H]^+$. $C_{23}H_{25}N_4O_3$. Calculated, *m*/*z*: 405.1927.

Step 2. **2-((1*S*)-1-Amino-2,2-dimethylpropyl)-5-(phenylamino)-1,3-oxazole-4-carbonitrile (19)** was obtained from benzyl *N*-{(1*S*)-1-[4-cyano-5-(phenylamino)-1,3-oxazol-2-yl]-2,2-dimethylpropyl}carbamate (step 1) (1.05 g, 2.6 mmol), 33% HBr solution in AcOH (27 ml, 156 mmol), and morpholine (2.3 ml, 26.0 mmol) in 1,4-dioxane following the general method II. The crude amine **19** was used in the next step without additional purification. Yield 390 mg (56%), yellow oil.



***tert*-Butyl [(1*S*)-1-({(1*S*)-1-[4-cyano-5-(phenylamino)-1,3-oxazol-2-yl]-2,2-dimethylpropyl}carbamoyl)but-3-en-1-yl]carbamate (20)** was obtained from aminooxazole **19** (390 mg, 1.4 mmol), EDC·HCl (553 mg, 2.9 mmol), and acid **16A** (466 mg, 2.2 mmol) in anhydrous pyridine following the general method III. Yield 585 mg (87%), colorless oil, $[\alpha]_D^{20}$ –28.0° (*c* 1.0, $CHCl_3$). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 7.39–7.32 (2H, m, H Ph); 7.23–7.16 (2H, m, H Ph); 7.14–7.08 (1H, m, H Ph); 5.75 (1H, ddd, *J* = 17.3, *J* = 10.0, *J* = 7.2, C<u>H</u>=CH_2); 5.20–5.10 (2H, m, CH=C<u>H</u>$_2$); 4.94 (1H, d, *J* = 9.3, CH–*t*-Bu); 4.18–4.11 (1H, m, C<u>H</u>NHCOO); 2.59–2.45 (2H, m, C<u>H</u>$_2$–CH=); 1.45 (9H, s, $C(CH_3)_3$); 1.00 (9H, s, $C(CH_3)_3$). ^{13}C NMR spectrum ($CDCl_3$), δ, ppm: 171.3; 170.3; 156.8; 153.7; 137.6; 133.1; 129.6; 124.1; 119.3; 118.4; 113.8; 89.3; 80.6; 55.4; 53.9; 35.8; 28.3; 26.2. Found, *m*/*z*: 490.2426 $[M+Na]^+$. $C_{25}H_{33}NaN_5O_4$. Calculated, *m*/*z*: 490.2430.

***tert*-Butyl *N*-{(1*S*)-1-[((1*S*)-1-{4-cyano-5-[phenyl(prop-2-en-1-yl)amino]-1,3-oxazol-2-yl}-2,2-dimethylpropyl)-carbamoyl]but-3-en-1-yl}carbamate (8cA)**. 60% NaH in mineral oil (116 mg, 2.9 mmol) was added to a solution of phenylaminooxazole **20** (450 mg, 1.0 mmol) in anhydrous DMF (10 ml) at 0°C. After the hydrogen evolution was ceased, the resulting yellow suspension was stirred for 15 min, whereupon allyl bromide (0.08 ml, 1.0 mmol) was added dropwise. The resulting solution was stirred for 1 h at 0°C, quenched by the addition of H_2O, and extracted with EtOAc (3×50 ml). The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4, and

concentrated under reduced pressure. The residue was purified by column chromatography on silica gel using gradient elution from pure hexane to 30% EtOAc in hexane to afford the title compound **8cA**. Yield 185 mg (38%), yellow oil, $[\alpha]_D^{20}$ –36.0° (*c* 1.0, $CHCl_3$). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 7.45–7.27 (3H, m, H Ph); 7.25–7.08 (2H, m, H Ph); 6.96–6.90 (1H, m, NH); 5.98–5.88 (1H, m, C<u>H</u>=CH_2); 5.79–5.71 (1H, m, C<u>H</u>=CH_2); 5.30–5.23 (2H, m, CH=C<u>H</u>$_2$); 5.19–5.11 (2H, m, CH=C<u>H</u>$_2$); 5.01–4.92 (1H, m, C<u>H</u>NHCOO); 4.86 (1H, d, *J* = 9.4, C<u>H</u>–oxazole); 4.40 (2H, ddt, *J* = 16.5, *J* = 6.8, *J* = 1.4, C<u>H</u>$_2$–CH=); 4.18–4.08 (1H, m, NH); 2.58–2.44 (2H, m, C<u>H</u>$_2$–CH=); 1.45 (9H, s, $C(CH_3)_3$); 0.95 (9H, s, $C(CH_3)_3$). ^{13}C NMR spectrum ($CDCl_3$), δ, ppm: 171.3; 158.7; 155.8; 153.1; 141.6; 133.2; 132.1; 129.8; 127.5; 125.2; 119.4; 118.8; 114.0; 88.7; 80.7; 55.2 (2C); 54.0; 36.0; 35.9; 28.4; 26.3. Found, *m*/*z*: 508.2921 $[M+H]^+$. $C_{28}H_{38}N_5O_4$. Calculated, *m*/*z*: 508.2924.

***tert*-Butyl *N*-((4*Z*,7*S*,10*S*)-10-*tert*-butyl-13-cyano-2-methyl-8-oxo-14-oxa-2,9,12-triazabicyclo[9.2.1]tetradeca-1(13),4,11-trien-7-yl)carbamate (21)**, obtained as a mixture of *E*/*Z*-isomers in 59:41 ratio. Compound **21** was synthesized from amide **8aA** (350 mg, 0.8 mmol) and ruthenium metathesis catalyst **Ru4** (29 mg, 0.04 mmol) in anhydrous CH_2Cl_2 following the general method IV. Purification of the crude product by column chromatography on silica gel using gradient elution from pure CH_2Cl_2 to 5% MeOH in CH_2Cl_2 afforded product **21**. Yield 220 mg (65%), yellow amorphous solid. Individual isomers were obtained after separation by silica gel column chromatography using CH_2Cl_2–EtOAc, 9:1 as eluent.

Compound (*Z*)-21. Yield 121 mg (37%), white amorphous solid, $[\alpha]_D^{20}$ –294.8° (*c* 1.0, MeOH). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 6.36 (1H, d, *J* = 6.9, N<u>H</u>–CO); 5.64–5.53 (2H, m, NH, CH=); 5.43–5.33 (1H, m, CH=); 4.44 (1H, d, *J* = 6.9, C<u>H</u>–$C(CH_3)_3$); 4.41–4.30 (1H, m, NH–C<u>H</u>–CH_2); 4.15–4.05 (1H, m, CH_2); 3.67–3.59 (1H, m, CH_2); 3.23 (3H, s, CH_3); 2.55–2.47 (1H, m, CH_2); 2.39–2.28 (1H, m, CH_2); 1.39 (9H, s, $C(CH_3)_3$); 1.03 (9H, s, $C(CH_3)_3$). ^{13}C NMR spectrum ($CDCl_3$), δ, ppm: 172.2; 160.5; 155.2; 150.9; 130.4; 128.0; 115.9; 86.2; 80.0; 57.7; 57.0; 53.8; 39.3; 36.4; 33.5; 28.3; 26.5. Found, *m*/*z*: 418.2494 $[M+H]^+$. $C_{21}H_{32}N_5O_4$. Calculated, *m*/*z*: 418.2454.

Compound (*E*)-21. Yield 84 mg (26%), beige amorphous solid, $[\alpha]_D^{20}$ –226.7° (*c* 0.6, MeOH). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 5.96 (1H, d, *J* = 6.2, NHCO); 5.74 (1H, t, *J* = 11.1, CH=); 5.65 (1H, d, *J* = 6.2, C<u>H</u>–$C(CH_3)_3$); 5.59–5.52 (1H, m, CH=); 5.28 (1H, s, NH); 4.54–4.43 (3H, m, CH_2, NH–C<u>H</u>–CH_2); 3.23 (3H, s, CH_3); 2.84 (1H, t, *J* = 13.1, CH_2); 2.60–2.52 (1H, m, CH_2); 1.41 (9H, s, $C(CH_3)_3$); 1.05 (9H, s, $C(CH_3)_3$). ^{13}C NMR spectrum ($CDCl_3$), δ, ppm: 171.6; 160.1; 155.0; 151.1; 128.2; 123.8; 115.8; 86.1; 80.0; 57.8; 52.6; 49.8; 37.8; 33.7; 31.1; 28.3; 26.5. Found, *m*/*z*: 418.2489 $[M+H]^+$. $C_{21}H_{32}N_5O_4$. Calculated, *m*/*z*: 418.2441.

***tert*-Butyl *N*-((7*S*,10*S*)-10-*tert*-butyl-13-cyano-2-methyl-8-oxo-14-oxa-2,9,12-triazabicyclo[9.2.1]tetradeca-1(13),11-dien-7-yl)carbamate (7aA)** was obtained by the hydrogenation of alkene (*Z*)-**21** (88 mg, 0.2 mmol) in the presence of 10% Pd on carbon (23 mg, 0.02 mmol) following the general method V. Yield 85 mg (96%), yellow oil, $[\alpha]_D^{20}$ –63.0° (*c* 1.0, $CHCl_3$). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 5.88 (1H, d, *J* = 7.6, NHCO); 5.51 (1H, d, *J* = 7.6, C<u>H</u>$C(CH_3)_3$); 4.59–4.48 (2H, m, C<u>H</u>CH_2, NHCOO); 3.84 (1H, t, *J* = 14.5, CH_2); 3.17 (3H, s, CH_3); 3.00 (1H, dt, *J* = 14.5, *J* = 3.1, CH_2); 2.02–1.76 (3H, m, CH_2); 1.61–1.49 (1H, m, CH_2); 1.48–1.36 (10H, m, CH_2, $C(CH_3)_3$); 1.33–1.21 (1H, m, CH_2); 1.06 (9H, s, $C(CH_3)_3$). ^{13}C NMR spectrum ($CDCl_3$), δ, ppm: 172.9; 160.1; 155.3; 150.1; 116.4; 83.5; 79.9; 58.9; 53.4; 49.1; 36.4; 33.6; 31.0; 28.5; 26.7; 25.4. Found, *m*/*z*: 442.2415 $[M+Na]^+$. $C_{21}H_{33}NaN_5O_4$. Calculated, *m*/*z*: 442.2430.

***tert*-Butyl *N*-((8*S*,11*S*)-11-*tert*-butyl-14-cyano-2-methyl-9-oxo-15-oxa-2,10,13-triazabicyclo[10.2.1]pentadeca-1(14),12-dien-8-yl)carbamate (7aB)** was obtained in a two-step sequence from amide **8aB** (200 mg, 0.4 mmol) and ruthenium metathesis catalyst **Ru4** (16 mg, 0.02 mmol) in anhydrous CH_2Cl_2 following the general method IV. The crude mixture of *E*/*Z*-isomers in 2:1 ratio of the resulting macrocycle was hydrogenated in the presence of 10% Pd on carbon (46 mg, 0.04 mmol) following the general method V. Yield in two steps 90 mg (48%), white amorphous solid, $[\alpha]_D^{20}$ –59.7° (*c* 1.0, $CHCl_3$). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 6.17 (1H, d, *J* = 9.3, NHCO); 5.50 (1H, d, *J* = 7.5, NHCOO); 4.88 (1H, d, *J* = 9.3, C<u>H</u>$C(CH_3)_3$); 4.36–4.27 (1H, m, C<u>H</u>CH_2); 3.63–3.56 (1H, m, CH_2); 3.19–3.07 (4H, m, CH_3, CH_2); 1.94–1.83 (1H, m, CH_2); 1.64–1.48 (2H, m, CH_2); 1.44–1.30 (13H, m, CH_2, $C(CH_3)_3$); 1.15–1.03 (10H, m, CH_2, $C(CH_3)_3$). ^{13}C NMR spectrum ($CDCl_3$), δ, ppm: 171.7; 160.2; 155.3; 149.7; 116.7; 83.3; 79.9; 55.7; 53.9; 49.5; 36.1; 33.8; 32.9; 28.5; 26.5; 25.8; 24.6; 20.8. Found, *m*/*z*: 456.2574 $[M+Na]^+$. $C_{22}H_{35}NaN_5O_4$. Calculated, *m*/*z*: 456.2587.

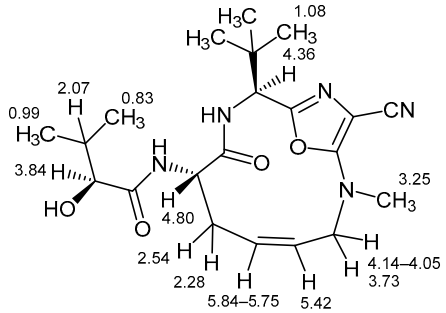
***tert*-Butyl *N*-((9*S*,12*S*)-12-*tert*-butyl-15-cyano-2-methyl-10-oxo-16-oxa-2,11,14-triazabicyclo[11.2.1]hexadeca-1(15),13-dien-9-yl)carbamate (7aC)** was obtained in a two-step sequence from amide **8aC** (350 mg, 0.7 mmol) and ruthenium metathesis catalyst **Ru4** (27 mg, 0.04 mmol) in anhydrous CH_2Cl_2 following the general method IV. The crude mixture of *E*/*Z*-isomers in 2:1 ratio of the resulting macrocycle was hydrogenated in the presence of 10% Pd on carbon (88 mg, 0.08 mmol) following the general method V. Yield in two steps 161 mg (44%), white amorphous solid, $[\alpha]_D^{20}$ –144.6° (*c* 1.0, MeOH). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 6.54 (1H, br. s, NHCO); 5.25 (1H, br. s, NHCOO); 4.82 (1H, d, *J* = 9.2, C<u>H</u>$C(CH_3)_3$); 4.19–4.10 (1H, m, C<u>H</u>CH_2); 3.68–3.58 (1H, m, CH_2); 3.19–3.11 (4H, m, CH_3, CH_2); 1.90–1.76 (2H, m, CH_2); 1.70–1.58 (2H, m, CH_2); 1.48–1.38 (11H, m, CH_2, $C(CH_3)_3$); 1.38–1.28 (2H, m, CH_2); 1.23–1.15 (2H, m, CH_2); 1.03 (9H, s, $C(CH_3)_3$). ^{13}C NMR spectrum ($CDCl_3$), δ, ppm: 171.4; 160.2; 155.6; 149.8; 116.4; 84.0; 80.1; 55.7; 54.0; 49.5; 35.8; 34.8; 31.3; 28.3; 26.3; 26.0; 25.2; 24.8; 24.4. Found, *m*/*z*: 448.2924 $[M+H]^+$. $C_{23}H_{38}N_5O_4$. Calculated, *m*/*z*: 448.2924.

***tert*-Butyl *N*-((7*S*,10*S*)-2-benzyl-10-*tert*-butyl-13-cyano-8-oxo-14-oxa-2,9,12-triazabicyclo[9.2.1]tetradeca-1(13),11-dien-7-yl)carbamate (7bA)** was obtained in a two-step sequence from amide **8bA** (280 mg, 0.54 mmol) and ruthenium metathesis catalyst **Ru4** (20 mg, 0.03 mmol) in anhydrous CH_2Cl_2 following the general method IV. The crude mixture of *E*/*Z*-isomers in 2:1 ratio of the resulting macrocycle was hydrogenated in the presence of 10% Pd

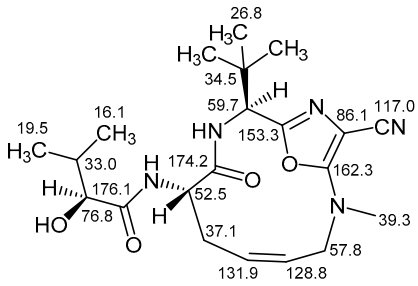
on carbon (57 mg, 0.05 mmol) following the general method V. Yield in two steps 90 mg (34%), white amorphous solid, $[\alpha]_D^{20}$ –30.3° (*c* 1.0, $CHCl_3$). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 7.40–7.26 (5H, m, H Ph); 5.87 (1H, d, *J* = 7.5, NHCO); 5.51 (1H, d, *J* = 7.5, C<u>H</u>C$(CH_3)_3$); 4.81 (1H, d, *J* = 15.6, NHCOO); 4.60–4.43 (3H, m, C<u>H</u>$_2$Ph, C<u>H</u>CH_2); 3.73 (1H, t, *J* = 15.6, CH_2); 3.05 (1H, dt, *J* = 15.6, *J* = 3.3, CH_2); 2.05–1.96 (1H, m, CH_2); 1.88–1.70 (2H, m, CH_2); 1.58–1.37 (11H, m, CH_2, C$(CH_3)_3$); 1.24–1.12 (1H, m, CH_2); 1.08 (9H, s, C$(CH_3)_3$). ^{13}C NMR spectrum ($CDCl_3$), δ, ppm: 172.9; 160.1; 155.3; 150.0; 135.6; 129.1; 128.4; 127.9; 116.0; 83.7; 79.9; 58.8; 53.4; 52.3; 46.1; 33.7; 31.1; 28.5; 26.7; 25.8; 18.2. Found, *m*/*z*: 496.2921 $[M+H]^+$. $C_{27}H_{38}N_5O_4$. Calculated, *m*/*z*: 496.2924.

***tert*-Butyl *N*-((7*S*,10*S*)-10-*tert*-butyl-13-cyano-8-oxo-2-phenyl-14-oxa-2,9,12-triazabicyclo[9.2.1]tetradeca-1(13),11-dien-7-yl)carbamate (7cA)** was obtained in a two-step sequence from amide **8cA** (185 mg, 0.4 mmol) and ruthenium metathesis catalyst **Ru4** (14 mg, 0.02 mmol) in anhydrous CH_2Cl_2 following the general method IV. The crude mixture of *E*/*Z*-isomers in 2:1 ratio of the resulting macrocycle was hydrogenated in the presence of 10% Pd on carbon (39 mg, 0.04 mmol) following the general method V. Yield in two steps 82 mg (47%), white amorphous solid, $[\alpha]_D^{20}$ –126.9° (*c* 0.6, $CHCl_3$). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 7.49–7.27 (5H, m, H Ph); 5.91 (1H, d, *J* = 7.5, NHCO); 5.56 (1H, d, *J* = 7.5, C<u>H</u>C$(CH_3)_3$); 4.62–4.57 (2H, m, C<u>H</u>CH_2, NHCOO); 4.12 (1H, t, *J* = 14.4, CH_2); 3.51 (1H, dt, *J* = 14.4, *J* = 3.2, CH_2); 2.13–2.01 (1H, m, CH_2); 1.98–1.85 (2H, m, CH_2); 1.68–1.56 (1H, m, CH_2); 1.43 (9H, s, C$(CH_3)_3$); 1.32–1.23 (1H, m, CH_2); 1.19–1.06 (10H, m, CH_2, C$(CH_3)_3$). ^{13}C NMR spectrum ($CDCl_3$), δ, ppm: 172.9; 159.3; 155.2; 150.4; 139.9; 130.1; 128.7; 127.5; 113.6; 85.8; 79.8; 58.8; 53.3; 50.1; 33.6; 31.1; 28.4; 26.6; 26.2; 17.9. Found, *m*/*z*: 482.2759 $[M+H]^+$. $C_{26}H_{36}N_5O_4$. Calculated, *m*/*z*: 482.2767.

(2*S*)-*N*-((4*Z*,7*S*,10*S*)-10-*tert*-Butyl-13-cyano-2-methyl-8-oxo-14-oxa-2,9,12-triazabicyclo[9.2.1]tetradeca-1(13),4,11-trien-7-yl)-2-hydroxy-3-methylbutanamide ((*Z*)-3) was obtained from compound (*Z*)-**21** (110 mg, 0.3 mmol), CF_3CO_2H (1.0 ml, 13.2 mmol), (*S*)-2-hydroxy-3-methylbutanoic acid (47 mg, 0.4 mmol), EDC·HCl (152 mg, 0.8 mmol), HOBt (107 mg, 0.8 mmol), and DIPEA (0.28 ml, 1.6 mmol) following the general method VI. Yield 60 mg (54%), white amorphous solid, $[\alpha]_D^{20}$ –264.1° (*c* 0.7, CD_3OD). ^{1}H NMR spectrum (CD_3OD), δ, ppm (*J*, Hz): 5.84–5.75 (1H, m, CH=); 5.42 (1H, dddd, *J* = 11.4, *J* = 10.2, *J* = 2.8, *J* = 2.8, CH=); 4.80 (1H, dd, *J* = 11.4, *J* = 5.4, C<u>H</u>CH_2); 4.36 (1H, s, C<u>H</u>C$(CH_3)_3$); 4.14–4.05 (1H,



(Z)-**3**



(Z)-**3**

m, CH_2); 3.84 (1H, d, *J* = 3.5, C<u>H</u>OH); 3.73 (1H, dq, *J* = 14.7, *J* = 2.8, CH_2); 3.25 (3H, s, NCH_3); 2.54 (1H, dddd, *J* = 13.1, *J* = 5.4, *J* = 5.4, *J* = 2.8, CH_2); 2.28 (1H, ddd, *J* = 13.1, *J* = 11.4, *J* = 11.4, CH_2); 2.07 (1H, sept d, *J* = 6.9, *J* = 3.4, $CH(CH_3)_2$); 1.08 (9H, s, C$(CH_3)_3$); 0.99 (3H, d, *J* = 6.9, C<u>H</u>$_3$CH); 0.83 (3H, d, *J* = 6.9, C<u>H</u>$_3$CH). ^{13}C NMR spectrum (CD_3OD), δ, ppm: 176.1; 174.2; 162.3; 153.3; 131.9; 128.8; 117.0; 86.1; 76.8; 59.7; 57.8; 52.5; 39.3; 37.1; 34.5; 33.0; 26.8; 19.5; 16.1. Found, *m*/*z*: 418.2455 $[M+H]^+$. $C_{21}H_{32}N_5O_4$. Calculated, *m*/*z*: 418.2454.

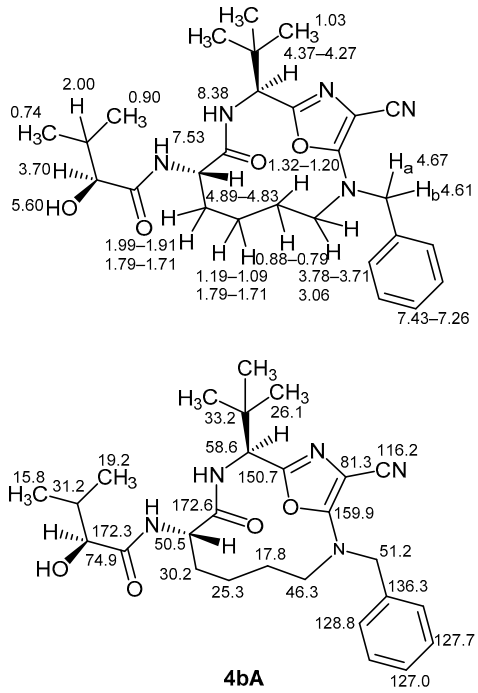
(2*S*)-*N*-((4*E*,7*S*,10*S*)-10-*tert*-Butyl-13-cyano-2-methyl-8-oxo-14-oxa-2,9,12-triazabicyclo[9.2.1]tetradeca-1(13),4,11-trien-7-yl)-2-hydroxy-3-methylbutanamide ((*E*)-3) was obtained from compound (*E*)-**21** (83 mg, 0.2 mmol), CF_3CO_2H (0.76 ml, 9.9 mmol), (*S*)-2-hydroxy-3-methylbutanoic acid (35 mg, 0.3 mmol), EDC·HCl (114 mg, 0.6 mmol), HOBt (81 mg, 0.6 mmol), and DIPEA (0.21 ml, 1.2 mmol) following the General method VI. Yield 48 mg (58%), beige amorphous solid, $[\alpha]_D^{20}$ –190.7° (*c* 1.0, MeOH). ^{1}H NMR spectrum (CD_3OD), δ, ppm (*J*, Hz): 5.71–5.57 (2H, m, 2CH=); 4.92 (1H, dd, *J* = 5.3, *J* = 2.4, C<u>H</u>CH_2); 4.59–4.49 (1H, m, CH_2); 3.89 (1H, d, *J* = 3.3, C<u>H</u>OH); 3.38 (1H, s, C<u>H</u>C$(CH_3)_3$); 3.35 (1H, s, CH_2); 3.25 (3H, s, NCH_3); 3.14–3.04 (1H, m, CH_2); 2.58–2.47 (1H, m, CH_2); 2.18–2.07 (1H, m, C<u>H</u>$(CH_3)_2$); 1.09 (9H, s, C$(CH_3)_3$); 1.01 (3H, d, *J* = 6.9, C<u>H</u>$_3$CH); 0.85 (3H, d, *J* = 6.9, C<u>H</u>$_3$CH). ^{13}C NMR spectrum (CD_3OD), δ, ppm: 174.6; 172.8; 160.8; 151.8; 130.5; 127.4; 115.5; 84.7; 75.3; 58.3; 56.3; 51.0; 37.9; 35.7; 33.1; 31.5; 25.3; 18.1; 14.7. Found, *m*/*z*: 418.2477 $[M+H]^+$. $C_{21}H_{32}N_5O_4$. Calculated, *m*/*z*: 418.2454.

(2*S*)-*N*-((7*S*,10*S*)-10-*tert*-Butyl-13-cyano-2-methyl-8-oxo-14-oxa-2,9,12-triazabicyclo[9.2.1]tetradeca-1(13),11-dien-7-yl)-2-hydroxy-3-methylbutanamide (4aA) was obtained from compound **7aA** (80 mg, 0.2 mmol), CF_3CO_2H (0.73 ml, 9.5 mmol), (*S*)-2-hydroxy-3-methylbutanoic acid (34 mg, 0.3 mmol), EDC·HCl (108 mg, 0.6 mmol), HOBt (76 mg, 0.6 mmol), and DIPEA (0.2 ml, 1.1 mmol) following the General method VI. Yield 39 mg (50%), white amorphous solid, $[\alpha]_D^{20}$ –109.5° (*c* 1.0, MeOH). ^{1}H NMR spectrum (CD_3OD), δ, ppm (*J*, Hz): 4.41 (1H, s, C<u>H</u>C$(CH_3)_3$); 3.95–3.86 (1H, m, C<u>H</u>CH_2); 3.86 (1H, d, *J* = 3.2, C<u>H</u>OH); 3.18 (3H, s, NCH_3); 3.09 (1H, dt, *J* = 14.7, *J* = 3.2, CH_2); 2.16–2.02 (2H, m, CH_2, C<u>H</u>$(CH_3)_2$); 2.00–1.86 (2H, m, CH_2); 1.50–1.37 (1H, m, CH_2); 1.32–1.21 (1H, m, CH_2); 1.09 (9H, s, C$(CH_3)_3$); 1.05–1.01 (1H, m, CH_2); 1.00 (3H, d, *J* = 6.9, C<u>H</u>$_3$CH); 0.93–0.85 (1H, m, CH_2); 0.83 (3H, d, *J* = 6.9, C<u>H</u>$_3$CH). ^{13}C NMR spectrum (CD_3OD), δ, ppm: 175.8; 174.7; 161.6; 152.5; 117.4; 83.0; 76.8; 60.7; 52.6; 49.8; 36.4; 34.4; 32.9; 31.4; 26.7; 26.3; 19.6; 19.1. Found, *m*/*z*: 420.2599 $[M+H]^+$. $C_{21}H_{34}N_5O_4$. Calculated, *m*/*z*: 420.2611.

(2*S*)-*N*-((8*S*,11*S*)-11-*tert*-Butyl-14-cyano-2-methyl-9-oxo-15-oxa-2,10,13-triazabicyclo[10.2.1]pentadeca-1(14),12-dien-8-yl)-2-hydroxy-3-methylbutanamide (4aB) was obtained from compound **7aB** (90 mg, 0.2 mmol), CF_3CO_2H (1.0 ml, 12.5 mmol), (*S*)-2-hydroxy-3-methylbutanoic acid (37 mg, 0.3 mmol), EDC·HCl (119 mg, 0.6 mmol), HOBt (84 mg, 0.6 mmol), and DIPEA (0.2 ml, 1.2 mmol) following the general method VI. Yield 41 mg (46%), white amorphous solid, $[\alpha]_D^{20}$ –88.9° (*c* 1.1, MeOH). ^{1}H NMR spectrum (CD_3OD), δ, ppm (*J*, Hz): 4.79 (1H, s, C<u>H</u>C(CH$_3$)$_3$); 4.59 (1H, t, *J* = 5.3, C<u>H</u>CH$_2$); 3.88 (1H, d, *J* = 3.3, C<u>H</u>OH); 3.58–3.48 (1H, m, CH$_2$); 3.41–3.34 (1H, m, CH$_2$); 3.19 (3H, s, NCH$_3$); 2.15–2.02 (1H, m, C<u>H</u>(CH$_3$)$_2$); 1.79–1.71 (2H, m, CH$_2$); 1.70–1.56 (2H, m, CH$_2$); 1.49–1.37 (2H, m, CH$_2$); 1.36–1.18 (2H, m, CH$_2$); 1.15 (9H, s, C(CH$_3$)$_3$); 1.02 (3H, d, *J* = 6.9, C<u>H</u>$_3$CH); 0.86 (3H, d, *J* = 6.9, C<u>H</u>$_3$CH). ^{13}C NMR spectrum (CD_3OD), δ, ppm: 175.9; 173.7; 161.7; 151.5; 117.6; 83.4; 76.8; 57.6; 53.3; 50.3; 36.2; 34.6; 33.4; 33.0; 26.8; 26.7; 25.8; 21.9; 19.6; 16.1. Found, *m*/*z*: 434.2772 $[M+H]^+$. $C_{22}H_{36}N_5O_4$. Calculated, *m*/*z*: 434.2767.

(2*S*)-*N*-((9*S*,12*S*)-12-*tert*-Butyl-15-cyano-2-methyl-10-oxo-16-oxa-2,11,14-triazabicyclo[11.2.1]hexadeca-1(15),13-dien-9-yl)-2-hydroxy-3-methylbutanamide (4aC) was obtained from compound **7aC** (113 mg, 0.3 mmol), CF_3CO_2H (1.2 ml, 15.1 mmol), (*S*)-2-hydroxy-3-methylbutanoic acid (45 mg, 0.4 mmol), EDC·HCl (146 mg, 0.8 mmol), HOBt (103 mg, 0.8 mmol), and DIPEA (0.3 ml, 1.5 mmol) following the general method VI. Yield 56 mg (49%), white amorphous solid, $[\alpha]_D^{20}$ –183.4° (*c* 1.0, $CHCl_3$). ^{1}H NMR spectrum (CD_3OD), δ, ppm (*J*, Hz): 4.84 (1H, s, C<u>H</u>C(CH$_3$)$_3$); 4.57 (1H, dd, *J* = 10.4, *J* = 4.8, C<u>H</u>CH$_2$); 3.86 (1H, d, *J* = 3.7, C<u>H</u>OH); 3.83–3.74 (1H, m, CH$_2$); 3.34–3.25 (1H, m, CH$_2$); 3.18 (3H, s, NCH$_3$); 2.13–2.05 (1H, m, C<u>H</u>(CH$_3$)$_2$); 1.87–1.69 (3H, m, CH$_2$); 1.69–1.61 (2H, m, CH$_2$); 1.45–1.36 (2H, m, CH$_2$); 1.34–1.23 (1H, m, CH$_2$); 1.19–1.10 (2H, m, CH$_2$); 1.08 (9H, s, C(CH$_3$)$_3$); 1.01 (3H, d, *J* = 6.9, C<u>H</u>$_3$CH); 0.86 (3H, d, *J* = 6.9, C<u>H</u>$_3$CH). ^{13}C NMR spectrum (CD_3OD), δ, ppm: 175.9; 173.6; 162.0; 151.6; 117.4; 84.2; 76.8; 57.1; 53.4; 50.5; 36.1; 36.0; 33.3; 33.0; 27.3; 26.8; 26.2; 25.7; 25.4; 19.6; 16.2. Found, *m*/*z*: 448.2937 $[M+H]^+$. $C_{23}H_{38}N_5O_4$. Calculated, *m*/*z*: 448.2924.

(2*S*)-*N*-((7*S*,10*S*)-2-Benzyl-10-*tert*-butyl-13-cyano-8-oxo-14-oxa-2,9,12-triazabicyclo[9.2.1]tetradeca-1(13),11-dien-7-yl)-2-hydroxy-3-methylbutanamide (4bA) was obtained from compound **7bA** (80 mg, 0.2 mmol), CF_3CO_2H (0.7 ml, 9.7 mmol), (*S*)-2-hydroxy-3-methylbutanoic acid (29 mg, 0.2 mmol), EDC·HCl (93 mg, 0.5 mmol), HOBt (66 mg, 0.5 mmol), and DIPEA (0.2 ml, 1.0 mmol) following the general method VI. Yield 25 mg (31%), white amorphous solid, $[\alpha]_D^{20}$ –52.0° (*c* 1.0, DMSO-d_6). ^{1}H NMR spectrum (DMSO-d_6), δ, ppm (*J*, Hz): 8.38 (1H, d, *J* = 6.7, NHCO); 7.53 (1H, d, *J* = 7.6, NHCOO); 7.43–7.26 (5H, m, H Ph); 5.60 (1H, d, *J* = 5.7, OH); 4.89–4.83 (1H, m, C<u>H</u>CH$_2$); 4.67 (1H, d, *J* = 17.4, C<u>H</u>$_2$Ph); 4.61 (1H, d, *J* = 16.8, C<u>H</u>$_2$Ph); 4.37–4.27 (1H, m, C<u>H</u>C(CH$_3$)$_3$); 3.78–3.71 (1H, m, CH$_2$N); 3.70 (1H, dd, *J* = 5.6, *J* = 3.2, C<u>H</u>OH); 3.06 (1H, d, *J* = 15.0, CH$_2$); 2.00 (1H, sept d, *J* = 6.8, *J* = 3.2, C<u>H</u>(CH$_3$)$_2$); 1.99–1.91 (1H, m, CH$_2$); 1.79–1.71 (2H, m, CH$_2$); 1.32–1.20 (1H, m, CH$_2$); 1.19–1.09 (1H, m, CH$_2$); 1.03 (9H, s, C(CH$_3$)$_3$); 0.90 (3H, d, *J* = 6.9, C<u>H</u>$_3$CH); 0.88–0.79 (1H, m, CH$_2$); 0.74 (3H, d, *J* = 6.9, C<u>H</u>$_3$CH). ^{13}C NMR spectrum (DMSO-d_6), δ, ppm: 172.6; 172.3; 159.9; 150.7; 136.3; 128.8; 127.7; 127.0; 116.2; 81.3; 74.9; 58.6; 51.2; 50.5; 46.3; 33.2; 31.2; 30.2; 26.1; 25.3; 19.2; 17.8; 15.8. Found, *m*/*z*: 496.2921 $[M+H]^+$. $C_{27}H_{38}N_5O_4$. Calculated, *m*/*z*: 496.2924.



(2*S*)-*N*-((7*S*,10*S*)-10-*tert*-Butyl-13-cyano-8-oxo-2-phenyl-14-oxa-2,9,12-triazabicyclo[9.2.1]tetradeca-1(13),11-dien-7-yl)-2-hydroxy-3-methylbutanamide (4cA) was obtained from compound **7cA** (80 mg, 0.2 mmol), CF_3CO_2H (0.6 ml, 8.3 mmol), (*S*)-(+)-2-hydroxy-3-methylbutanoic acid (30 mg, 0.3 mmol), EDC·HCl (96 mg, 0.5 mmol), HOBt (68 mg, 0.5 mmol), and DIPEA (0.2 ml, 1.0 mmol) following the general method VI. Yield 40 mg (50%), white amorphous solid, $[\alpha]_D^{20}$ –111.0° (*c* 1.0, MeOH). ^{1}H NMR spectrum (CD_3OD), δ, ppm (*J*, Hz): 7.55–7.46 (2H, m, H Ph); 7.44–7.37 (3H, m, H Ph); 4.97–4.90 (1H, m, C<u>H</u>C(CH$_3$)$_3$); 4.50 (1H, d, *J* = 6.7, CH$_2$); 4.28–4.18 (1H, m, C<u>H</u>CH$_2$); 3.90 (1H, d, *J* = 3.3, C<u>H</u>OH); 3.57 (1H, d, *J* = 15.0, CH$_2$); 2.24–2.08 (2H, m, CH$_2$); 2.08–1.96 (1H, m, CH$_2$); 1.96–1.85 (1H, m, CH$_2$); 1.58–1.46 (1H, m, C<u>H</u>(CH$_3$)$_2$); 1.36–1.24 (1H, m, CH$_2$); 1.24–1.17 (1H, m, CH$_2$); 1.14 (9H, s, C(CH$_3$)$_3$); 1.03 (3H, d, *J* = 7.0, C<u>H</u>$_3$CH); 0.87 (3H, d, *J* = 7.0, C<u>H</u>$_3$CH). ^{13}C NMR spectrum (CD_3OD), δ, ppm: 175.9; 175.0; 161.1; 153.0; 141.6; 131.1; 129.7; 128.8; 114.8; 85.5; 76.8; 60.9; 52.7; 51.2; 34.6; 32.9; 31.6; 27.1; 26.8; 19.6; 19.0; 16.2. Found, *m*/*z*: 482.2765 $[M+H]^+$. $C_{26}H_{36}N_5O_4$. Calculated, *m*/*z*: 482.2767.

Methyl 2-{2-[(1*S*)-1-((2*S*)-2-{[(*tert*-butoxy)carbonyl]-amino}pent-4-enamido)-2,2-dimethylpropyl]-5-[methyl-(prop-2-en-1-yl)amino]-1,3-oxazol-4-yl}-1,3-oxazole-4-carboxylate (23) was synthesized in a three-step sequence from bioxazole **22**.

Step 1. **Methyl 2-[2-((1*S*)-1-amino-2,2-dimethylpropyl)-5-bromo-1,3-oxazol-4-yl]-1,3-oxazole-4-carboxylate**. 33% HBr solution in AcOH (2.6 ml, 15.0 mmol, 20 equiv) was added to a solution of bioxazole **22** (370 mg, 0.8 mmol,

1 equiv) in 1,4-dioxane (2 ml). The reaction mixture was stirred at room temperature for 15 min. Then Et_2O (20 ml) was added to the reaction mixture and white precipitate was formed. The resulting suspension was decanted, and the precipitate was washed with Et_2O (2×40 ml) and evaporated under reduced pressure to provide crude oxazole as a yellow oil, yield 280 mg (85%), which was used in the next reaction without additional purification.

Step 2. **Methyl 2-{2-((1*S*)-1-amino-2,2-dimethylpropyl)-5-[methyl(prop-2-en-1-yl)amino]-1,3-oxazol-4-yl}-1,3-oxazole-4-carboxylate** was obtained from methyl 2-[2-((1*S*)-1-amino-2,2-dimethylpropyl)-5-bromo-1,3-oxazol-4-yl]-1,3-oxazole-4-carboxylate (step 1) (280 mg, 0.8 mmol), *N*-methylallylamine (0.1 ml, 1.3 mmol), and NEt_3 (0.3 ml, 1.9 mmol) in anhydrous DMF at 50°C following the general method I. The crude product was purified by column chromatography on silica gel using gradient elution from pure CH_2Cl_2 to 10% MeOH in CH_2Cl_2 to obtain product as a yellow oil, yield 110 mg (40%), which was used in the next reaction without additional purification.

Step 3. **Methyl 2-{2-[(1*S*)-1-((2*S*)-2-{[(*tert*-butoxy)carbonyl]amino}pent-4-enamido)-2,2-dimethylpropyl]-5-[methyl(prop-2-en-1-yl)amino]-1,3-oxazol-4-yl}-1,3-oxazole-4-carboxylate (23)** was obtained from methyl 2-{2-((1*S*)-1-amino-2,2-dimethylpropyl)-5-[methyl(prop-2-en-1-yl)amino]-1,3-oxazol-4-yl}-1,3-oxazole-4-carboxylate (step 2) (110 mg, 0.3 mmol), acid **16A** (102 mg, 0.5 mmol), and EDC·HCl (121 mg, 0.6 mmol) in anhydrous pyridine following the general method III. Yield 110 mg (64%), yellow oil, $[\alpha]_D^{20}$ –48.0° (*c* 1.0, $CHCl_3$). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 8.19 (1H, s, H oxazole); 6.90 (1H, d, *J* = 9.6, NHCO); 5.94–5.66 (2H, m, 2C<u>H</u>=CH_2); 5.26–5.05 (4H, m, 2CH=C<u>H</u>$_2$); 5.02–4.97 (1H, m, NHCOO); 4.96 (1H, d, *J* = 9.6, C<u>H</u>$C(CH_3)_3$); 4.19–4.04 (3H, m, C<u>H</u>CH_2, CH_2); 3.89 (3H, s, OCH_3); 3.10 (3H, s, NCH_3); 2.55–2.40 (2H, m, CH_2); 1.42 (9H, s, $C(CH_3)_3$); 0.98 (9H, s, $C(CH_3)_3$). ^{13}C NMR spectrum ($CDCl_3$), δ, ppm: 171.1; 162.1; 157.7; 155.4; 152.8; 142.7; 133.9; 133.3; 132.9; 119.1; 118.4; 103.3; 80.4; 60.5; 55.3; 54.0; 52.1; 37.7; 36.2; 28.4; 26.4; 26.3. Found, *m*/*z*: 546.2932 $[M+H]^+$. $C_{27}H_{40}N_5O_7$. Calculated, *m*/*z*: 546.2928.

Methyl 2-((7*S*,10*S*)-7-{[(*tert*-butoxy)carbonyl]amino}-10-*tert*-butyl-2-methyl-8-oxo-14-oxa-2,9,12-triazabicyclo[9.2.1]tetradeca-1(13),11-dien-13-yl)-1,3-oxazole-4-carboxylate (24). Alkene **23** (90 mg, 0.2 mmol, 1 equiv) was added to a solution of ruthenium metathesis catalyst **Ru4** (12 mg, 0.02 mmol, 0.1 equiv) in anhydrous CH_2Cl_2 (100 ml). After stirring at 50°C for 24 h, neat Ti(O*i*-Pr)$_4$ (0.05 ml, 0.2 mmol, 1 equiv) was added dropwise. The stirring was continued for 5 days at 60°C, whereupon the reaction mixture was cooled to room temperature and concentrated under reduced pressure. The crude unsaturated macrocycle (as a mixture of *E*/*Z*-isomers) was hydrogenated in the presence of 10% Pd on carbon (18 mg, 0.02 mmol) following the general method V to afford product **24** as a white amorphous solid, yield 33 mg (39%), $[\alpha]_D^{20}$ –160.0° (*c* 1.0, $CHCl_3$). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 8.17 (1H, s, H oxazole); 5.81 (1H, d, *J* = 7.5, NHCO); 5.51 (1H, d, *J* = 7.5, C<u>H</u>$C(CH_3)_3$); 4.60 (1H, d, *J* = 15.4, NHCOO); 4.59–4.53 (1H, m, C<u>H</u>CH_2); 3.89 (3H, s, OCH_3); 3.89–3.83 (1H, m, CH_2); 3.27 (3H, s, NCH_3); 3.05–3.00 (1H, m, CH_2); 2.08–1.95 (2H, m, CH_2); 1.93–1.83 (1H, m, CH_2); 1.55–1.38 (10H, m, CH_2, $C(CH_3)_3$); 1.29–1.18 (1H, m, CH_2); 1.08 (9H, s, $C(CH_3)_3$); 1.03–0.91 (1H, m, CH_2). ^{13}C NMR spectrum ($CDCl_3$), δ, ppm: 172.9; 162.2; 158.1; 155.3; 154.8; 150.3; 142.7; 133.5; 100.1; 79.9; 58.8; 53.4; 52.1; 49.4; 37.0; 33.8; 31.5; 28.5; 26.8; 25.1; 18.0. Found, *m*/*z*: 520.2772 $[M+H]^+$. $C_{25}H_{38}N_5O_7$. Calculated, *m*/*z*: 520.2771.

(2*S*)-*N*-{(7*S*,10*S*)-10-*tert*-Butyl-13-[4-(hydroxymethyl)-1,3-oxazol-2-yl]-2-methyl-8-oxo-14-oxa-2,9,12-triazabicyclo[9.2.1]tetradeca-1(13),11-dien-7-yl}-2-hydroxy-3-methylbutanamide (5). CF_3CH_2OH (0.1 ml) was added to a solution of compound **24** (25 mg, 0.05 mmol, 1 equiv) in anhydrous THF (1 ml). The flask was cooled to 0°C (crushed ice bath), and $LiBH_4$ (5 mg, 0.2 mmol, 5 equiv) was added in one portion. The resulting mixture was warmed to room temperature and stirred for 20 h, whereupon it was quenched by addition of aqueous saturated NH_4Cl solution and extracted with EtOAc (3×10 ml). The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to afford crude alcohol as colorless oil, yield 17 mg (72%), which was used in the next step without additional purification.

The title compound **5** was obtained from the crude alcohol (17 mg, 0.03 mmol), CF_3CO_2H (0.1 ml, 1.6 mmol), (*S*)-2-hydroxy-3-methylbutanoic acid (8 mg, 0.07 mmol), EDC·HCl (14 mg, 0.07 mmol), HOBt (15 mg, 0.08 mmol), and DIPEA (0.03 ml, 0.4 mmol) following the general method VI. Yield 10 mg (59%), white amorphous solid, $[\alpha]_D^{20}$ –154.8° (*c* 1.0, $CHCl_3$). ^{1}H NMR spectrum (CD_3OD), δ, ppm (*J*, Hz): 7.75 (1H, s, H oxazole); 4.53 (2H, s, C<u>H</u>$_2$OH); 4.51 (1H, s, C<u>H</u>$C(CH_3)_3$); 4.00–3.90 (1H, m, C<u>H</u>CH_2); 3.87 (1H, d, *J* = 3.3, C<u>H</u>OH); 3.17 (3H, s, NCH_3); 3.09 (1H, dt, *J* = 14.9, *J* = 3.3, CH_2); 2.18–1.88 (4H, m, CH_2, C<u>H</u>$(CH_3)_2$); 1.47–1.35 (1H, m, CH_2); 1.34–1.21 (2H, m, CH_2); 1.12 (9H, s, $C(CH_3)_3$); 1.05–0.93 (4H, m, C<u>H</u>$_3$CH, CH_2); 0.85 (3H, d, *J* = 6.8, C<u>H</u>$_3$CH). ^{13}C NMR spectrum (CD_3OD), δ, ppm: 175.9; 174.6; 158.8; 155.6; 152.8; 142.5; 135.6; 101.0; 76.8; 60.6; 57.3; 52.7; 50.0; 37.4; 34.6; 32.9; 31.8; 26.9; 26.3; 19.6; 19.1; 16.1. Found, *m*/*z*: 492.2818 $[M+H]^+$. $C_{24}H_{38}N_5O_6$. Calculated, *m*/*z*: 492.2822.

***tert*-Butyl *N*-((1*S*)-1-{4-(4-{[(*tert*-butyldimethylsilyl)oxy]methyl}-1,3-oxazol-2-yl)-5-[(3,5-difluorophenyl)(hydroxy)methyl]-1,3-oxazol-2-yl}-2,2-dimethylpropyl)carbamate (25)**. *n*-BuLi (1.6 M solution in hexane, 1.17 ml, 1.88 mmol, 2.05 equiv) was added gradually (within 20 min) to a solution of bioxazole (*S*)-**12**[4] (427 mg, 0.92 mmol, 1.0 equiv) in anhydrous Et_2O (9 ml) at –78°C. The color of the solution slowly changed from yellow to dark-brown. After 10 min of the stirring at –78°C, a solution of 3,5-difluorobenzaldehyde (313 mg, 2.20 mmol, 2.4 equiv) in anhydrous Et_2O (2 ml) was added dropwise. The resulting solution was stirred for 10 min at –78°C, slowly warmed to room temperature, stirred for 30 min, and cooled back to 0°C. Aqueous saturated NH_4Cl solution (20 ml) was added, and the mixture was extracted with Et_2O (3×20 ml). Combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel using gradient elution from 5% EtOAc in hexane to 50% EtOAc in hexane to afford alcohol **25** as

a mixture of diastereomers, yield 284 mg (51%), colorless oil, which was used in the next reaction without additional purification.

***tert*-Butyl *N*-{(1*S*)-1-[4-(4-{[(*tert*-butyldimethylsilyl)-oxy]methyl}-1,3-oxazol-2-yl)-5-(3,5-difluorobenzoyl)-1,3-oxazol-2-yl]-2,2-dimethylpropyl}carbamate (26)**. Dess–Martin periodinane (290 mg, 0.68 mmol, 1.5 equiv) was portionwise added to a solution of crude alcohol **25** (277 mg, 0.46 mmol, 1.0 equiv) in anhydrous CH_2Cl_2 (9 ml). The resulting white suspension was stirred at room temperature for 1 h, whereupon all volatiles were removed under reduced pressure. The residue was purified by flash chromatography using gradient elution from pure hexane to 60% EtOAc in hexane. Yield 215 mg (78%), white amorphous solid, $[\alpha]_D^{20}$ –42.0° (*c* 1.0, $CHCl_3$). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 7.61 (1H, dd, *J* = 1.4, *J* = 1.4, H Ar); 7.48–7.41 (2H, m, H Ar); 7.07 (1H, tt, *J* = 8.4, *J* = 2.4, H Ar); 5.45 (1H, d, *J* = 9.8, NH); 4.84 (1H, d, *J* = 9.8, C<u>H</u>NH); 4.74 (2H, s, C<u>H</u>$_2$OH); 1.43 (9H, s, OC(CH_3)$_3$); 1.05 (9H, s, C(CH_3)$_3$); 0.92 (9H, s, C(CH_3)$_3$); 0.10 (6H, s, 2CH_3Si). ^{13}C NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 179.3; 166.1; 163.0 (dd, *J* = 251.6, *J* = 11.8); 155.4; 153.8; 144.8; 144.2; 139.2 (t, *J* = 8.2); 136.7; 134.5; 112.9–112.5 (m); 109.1 (t, *J* = 25.2); 80.5; 59.2; 36.2; 31.7; 28.4; 26.4; 26.0; –5.3. Found, *m*/*z*: 606.2810 $[M+H]^+$. $C_{30}H_{42}F_2N_3O_6Si$. Calculated, *m*/*z*: 606.2811.

***tert*-Butyl *N*-((1*S*)-1-{4-(4-{[(*tert*-butyldimethylsilyl)-oxy]methyl}-1,3-oxazol-2-yl)-5-[1-(3,5-difluorophenyl)-1-hydroxybut-3-en-1-yl]-1,3-oxazol-2-yl}-2,2-dimethylpropyl)carbamate (27)**. Allylmagnesium bromide (1 M solution in Et_2O, 0.88 ml, 0.88 mmol, 2.5 equiv) was dropwise added to a solution of ketone **26** (214 mg, 0.35 mmol, 1.0 equiv) in anhydrous THF (5 ml) at –15°C. After stirring at –15°C for 30 min, the yellow solution was warmed to 0°C, quenched with aqueous saturated NH_4Cl solution, and extracted with EtOAc (3×15 ml). Combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel using gradient elution from pure hexane to 50% EtOAc in hexane to afford tertiary alcohol **27** as a mixture of diastereomers. Yield 188 mg (82%), colorless oil ^{1}H NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 8.27 (0.5H, s, NH); 8.24 (0.5H, s, NH); 7.59–7.54 (1H, m, H Ar); 7.02–6.94 (2H, m, H Ar); 6.69–6.62 (1H, m, H Ar); 5.86–5.70 (1H, m, C<u>H</u>=CH$_2$); 5.37–5.27 (1H, m, OH); 5.18–5.02 (2H, m, CH=C<u>H</u>$_2$); 4.78 (1H, d, *J* = 9.6, C<u>H</u>NH); 4.66–4.60 (2H, m, C<u>H</u>$_2$OH); 3.03 (1H, dt, *J* = 14.7, *J* = 7.5 C<u>H</u>$_2$); 2.93–2.79 (1H, m, C<u>H</u>$_2$); 1.44 (4.5H, s, OC(CH_3)$_3$); 1.43 (4.5H, s, OC(CH_3)$_3$); 1.03 (4.5H, s, C(CH_3)$_3$); 1.01 (4.5H, s, C(CH_3)$_3$); 0.92 (4.5H, s, C(CH_3)$_3$); 0.91 (4.5H, s, C(CH_3)$_3$); 0.12–0.08 (6H, m, 2CH_3Si). ^{13}C NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 166.5; 162.9 (dd, *J* = 250.6, *J* = 12.0); 156.1; 155.4; 149.3; 149.0; 141.8 (t, *J* = 8.0); 135.6; 132.2; 125.1; 119.2; 108.7–108.4 (m); 103.0 (t, *J* = 25.1); 80.3; 74.5; 58.1; 57.4; 46.0; 45.9; 35.8; 28.4; 26.4; 26.0; –5.2. Found, *m*/*z*: 648.3294 $[M+H]^+$. $C_{33}H_{48}F_2N_3O_6Si$. Calculated, *m*/*z*: 648.3280.

***tert*-Butyl *N*-{(1*S*)-1-[((1*S*)-1-{5-[1-(3,5-difluorophenyl)-1-hydroxybut-3-en-1-yl]-4-[4-(hydroxymethyl)-1,3-oxazol-2-yl]-1,3-oxazol-2-yl}-2,2-dimethylpropyl)carbamoyl]but-3-en-1-yl}carbamate (28)** was obtained in a two-step sequence from alcohol **27**.

Step 1. **1-{2-((1*S*)-1-Amino-2,2-dimethylpropyl)-4-[4-(hydroxymethyl)-1,3-oxazol-2-yl]-1,3-oxazol-5-yl}-1-(3,5-difluorophenyl)but-3-en-1-ol**. 4 M HCl solution in 1,4-dioxane (5.1 ml, 20.4 mmol, 50 equiv) was added to a solution of allylic alcohol **27** (265 mg, 0.41 mmol, 1.0 equiv) in 1,4-dioxane (9 ml). The resulting solution was stirred at room temperature for 18 h and concentrated under reduced pressure. The residue was dissolved in EtOAc (10 ml), washed with aqueous saturated $NaHCO_3$ solution and brine, then dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel using gradient elution from 1% MeOH in EtOAc to 10% MeOH in EtOAc to afford product as a mixture of diastereomers. Yield 123 mg (69%), yellow oil. ^{1}H NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 8.22 (1H, s, NH_2); 7.65–7.64 (1H, m, H Ar); 7.03–6.95 (2H, m, H Ar); 6.71–6.63 (1H, m, H Ar); 5.85–5.71 (1H, m, C<u>H</u>=CH$_2$); 5.18–5.05 (2H, m, CH=C<u>H</u>$_2$); 4.63 (2H, d, *J* = 1.1, C<u>H</u>$_2$OH); 3.86–3.84 (1H, m, C<u>H</u>NH$_2$); 3.08–2.98 (1H, m, C<u>H</u>$_2$); 2.93–2.82 (1H, m, CH$_2$); 1.99–1.70 (3H, br. s, 2OH, NH_2); 1.05–1.00 (9H, m, C(CH_3)$_3$). ^{13}C NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 165.9; 162.9 (dd, *J* = 248.7, *J* = 12.6); 161.7; 156.5; 155.4; 149.0 (dt, *J* = 7.9, *J* = 3.4); 141.1; 135.7; 132.2; 124.7; 119.4; 108.7–108.3 (m); 103.1 (dt, *J* = 25.4, *J* = 3.6); 74.5; 59.5; 57.1; 45.9; 35.7; 26.4. Found, *m*/*z*: 434.1896 $[M+H]^+$. $C_{22}H_{26}F_2N_3O_4$. Calculated, *m*/*z*: 434.1891.

Step 2. ***tert*-Butyl *N*-{(1*S*)-1-[((1*S*)-1-{5-[1-(3,5-difluorophenyl)-1-hydroxybut-3-en-1-yl]-4-[4-(hydroxymethyl)-1,3-oxazol-2-yl]-1,3-oxazol-2-yl}-2,2-dimethylpropyl)carbamoyl]but-3-en-1-yl}carbamate (28)** was obtained from 1-{2-((1*S*)-1-amino-2,2-dimethylpropyl)-4-[4-(hydroxymethyl)-1,3-oxazol-2-yl]-1,3-oxazol-5-yl}-1-(3,5-difluorophenyl)but-3-en-1-ol (step 1) (110 mg, 0.25 mmol), EDC·HCl (54 mg, 0.28 mmol), and acid **16A** (55 mg, 0.25 mmol) in anhydrous pyridine following the general method III. Purification by column chromatography on silica gel using gradient elution from 30% EtOAc in hexane to 70% EtOAc in hexane afforded product **28** as a mixture of diastereomers. Yield 114 mg (71%), yellow amorphous solid. ^{1}H NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 8.20 (1H, d, *J* = 12.4, NH); 7.66–7.64 (1H, m, H Ar); 7.19–7.04 (1H, m, NH); 7.02–6.94 (2H, m, H Ar); 6.72–6.60 (1H, m, H Ar); 5.87–5.66 (2H, m, 2C<u>H</u>=CH$_2$); 5.19–5.03 (5H, m, 2CH=C<u>H</u>$_2$, C<u>H</u>NH); 5.02–4.92 (1H, br. s, OH); 4.66–4.62 (2H, m, C<u>H</u>$_2$OH); 4.20–4.13 (1H, m, C<u>H</u>NH); 3.04 (1H, dt, *J* = 13.4, *J* = 6.4 CH$_2$); 2.88 (1H, td, *J* = 14.5, *J* = 6.7, CH$_2$); 2.58–2.46 (2H, m, CH$_2$, OH); 2.00 (1H, t, *J* = 6.0, CH$_2$); 1.44 (9H, s, OC(CH_3)$_3$); 1.03 (4.5H, s, C(CH_3)$_3$); 1.02 (4.5H, s, C(CH_3)$_3$). ^{13}C NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 163.0 (dd, *J* = 249.0, *J* = 12.6); 162.0; 161.9; 161.7; 156.4; 155.7; 149.0 (t, *J* = 8.0); 141.1; 135.7; 133.2; 132.1; 125.0; 119.5; 108.7–108.3 (m); 103.1 (dd, *J* = 25.5, *J* = 8.0); 80.6; 74.5; 57.1; 45.9; 45.8; 35.8; 28.4; 26.43; 26.40. Found, *m*/*z*: 631.2950 $[M+H]^+$. $C_{32}H_{41}F_2N_4O_7$. Calculated, *m*/*z*: 631.2943.

***tert*-Butyl *N*-{(2*S*,5*S*)-2-*tert*-butyl-10-(3,5-difluorophenyl)-10-hydroxy-12-[4-(hydroxymethyl)-1,3-oxazol-2-yl]-4-oxo-14-oxa-3,13-diazabicyclo[9.2.1]tetradeca-1(13),11-dien-**

5-yl}carbamate (29) was synthesized from amide **28** (102 mg, 0.16 mmol) and ruthenium metathesis catalyst **Ru4** (11.9 mg, 0.02 mmol) in anhydrous CH_2Cl_2 following the general method IV. The crude unsaturated macrocycle was obtained as a mixture of *E*/*Z*-isomers in 2:1 ratio after purification by column chromatography on silica gel using gradient elution from 25% EtOAc in hexane to 100% EtOAc. Yield 80 mg (82%), brown amorphous solid.

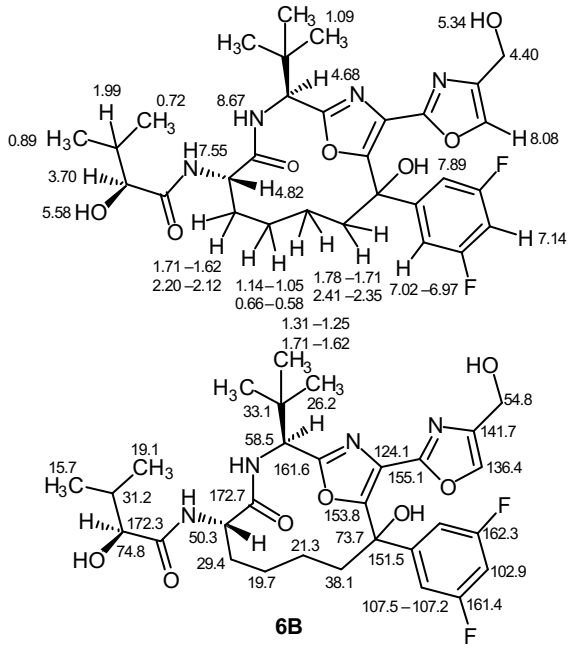
A solution of the crude macrocycle (80 mg, 0.13 mmol, 1.0 equiv) in MeOH (10 ml) was stirred for 5 days under H_2 pressure (5 atm) in the presence of 10% Pd on carbon (71 mg, 0.07 mmol, 0.5 equiv). The resulting black suspension was filtered through a pad of Celite®, and the filtrate was concentrated under reduced pressure. The residue was purified by column chromatography on silica gel using gradient elution from 30% EtOAc in hexane to 50% EtOAc in hexane to afford product **29** as a mixture of diastereomers. Yield 52 mg (65%), white amorphous solid. ^{1}H NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 8.06 (0.67H, s, NH); 7.65–7.62 (0.67H, m, H Ar); 7.61–7.59 (0.33H, m, H Ar); 7.41 (0.33H, s, NH); 6.94–6.86 (1.34H, m, H Ar); 6.85–6.79 (0.66H, m, H Ar); 6.69–6.60 (1H, m, H Ar); 6.26–6.19 (0.33H, m, NHCH); 6.16–6.09 (0.67H, m, NHCH); 5.54–5.42 (1H, m, CH_2OH); 5.18–5.11 (0.33H, m, CHNH); 4.90 (0.67H, d, *J* = 8.0, CHNH); 4.62 (1.34H, br. s, CH_2OH); 4.57–4.51 (0.67H, m, $CHCH_2$); 4.49 (0.66H, br. s, CH_2OH); 4.31–4.24 (0.33H, m, $CHCH_2$); 2.41–2.28 (1H, m, CH_2CH); 2.27–2.14 (2H, m, CH_2CH); 2.00–1.72 (4H, m, $2CH_2$, CH_2CH); 1.42 (2.97H, s, $OC(CH_3)_3$); 1.41 (6.03H, s, $OC(CH_3)_3$); 1.33–1.26 (1H, m, CH_2); 1.24 (2.97H, s, $C(CH_3)_3$); 1.17 (6.03H, s, $C(CH_3)_3$). ^{13}C NMR spectrum ($CDCl_3$), δ, ppm (*J*, Hz): 173.2; 163.0 (dd, *J* = 257.6, *J* = 12.6); 161.9; 161.2; 156.2; 155.3; 155.2; 151.4 (t, *J* = 7.4); 141.2; 135.7; 125.2; 107.8–107.4 (m); 102.9 (t, *J* = 25.3); 74.1; 58.9; 57.1; 53.4; 38.9; 33.6; 30.8; 28.5; 26.9; 21.9; 14.3. Found, *m*/*z*: 605.2771 $[M+H]^+$. $C_{30}H_{39}F_2N_4O_7$. Calculated, *m*/*z*: 605.2787.

(2*S*)-*N*-{(2*S*,5*S*)-2-*tert*-Butyl-10-(3,5-difluorophenyl)-10-hydroxy-12-[4-(hydroxymethyl)-1,3-oxazol-2-yl]-4-oxo-14-oxa-3,13-diazabicyclo[9.2.1]tetradeca-1(13),11-dien-5-yl}-2-hydroxy-3-methylbutanamide (diastereomers 6A and 6B). CF_3CO_2H (0.4 ml, 5.27 mmol, 65 equiv) was dropwise added to a solution of macrocycle **29** (49 mg, 0.081 mmol, 1.0 equiv) in anhydrous CH_2Cl_2 (3 ml) at 0°C. The yellow solution was warmed to room temperature and stirred for 3 h, whereupon all volatiles were removed under reduced pressure. The residue was dissolved in THF (0.5 ml) and MeOH (0.5 ml), and a solution of LiOH (10 mg, 0.40 mmol, 5 equiv) in H_2O (0.5 ml) was added dropwise. The resulting brown solution was stirred for 1 h at room temperature, and all volatiles were concentrated under reduced pressure. The residue was dissolved in Et_2O (10 ml) and washed with 1 M aqueous HCl (2×10 ml), brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. Crude amine was obtained as white amorphous solid, yield 41 mg (100%), which was used in the next reaction without additional purification.

(*S*)-2-Hydroxy-3-methylbutanoic acid (9.6 mg, 0.081 mmol, 1.05 equiv), EDC·HCl (15.6 mg, 0.081 mmol, 1.05 equiv), HOBt (11 mg, 0.081 mmol, 1.05 equiv), and DIPEA (40 μl, 0.23 mmol, 3.0 equiv) were added to a solution of crude amine (39 mg, 0.077 mmol, 1.0 equiv) in anhydrous DMF (5 ml). The yellow solution was stirred at room temperature for 18 h, whereupon H_2O was added, and the resulting mixture was extracted with EtOAc (3×10 ml). Combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was purified by reversed-phase column chromatography on silica gel using gradient elution from 30 to 50% MeCN in H_2O to afford individual diastereomers.

Diastereomer 6A. Yield 10.5 mg (23%), white amorphous solid, $[\alpha]_D^{20}$ –49.7° (*c* 0.7, MeOH). ^{1}H NMR spectrum (CD_3OD), δ, ppm (*J*, Hz): 8.62 (1H, d, *J* = 7.2, NH); 7.73 (1H, s, H Ar); 6.96–6.86 (2H, m, H Ar); 6.78 (1H, tt, *J* = 8.8, *J* = 2.3, H Ar); 4.99–4.92 (1H, m, CH); 4.74 (1H, dd, *J* = 7.2, *J* = 2.8, CH); 4.37 (2H, s, CH_2OH); 3.87 (1H, d, *J* = 3.4, CHOH); 2.96–2.87 (1H, m, CH_2CH); 2.57–2.40 (1H, m, CH_2CH); 2.16–1.80 (3H, m, CH_2CH, $CH(CH_3)_2$); 1.34–1.26 (4H, m, $2CH_2$); 1.22 (9H, s, $C(CH_3)_3$); 0.99 (3H, d, *J* = 6.9, CH_3CH); 0.82 (3H, d, *J* = 6.9, CH_3CH). ^{13}C NMR spectrum (CD_3OD), δ, ppm (*J*, Hz): 174.3; 173.4; 163.1 (dd, *J* = 248.0, *J* = 12.6); 156.2; 153.0; 142.9 (t, *J* = 8.1); 137.5; 137.0; 136.3; 126.5; 110.1–109.7 (m); 103.5 (t, *J* = 26.0); 76.5; 73.8; 59.5; 55.3; 51.3; 40.2; 32.9; 31.6; 29.6; 26.9; 22.7; 19.6; 18.2; 15.2. Found, *m*/*z*: 605.2789 $[M+H]^+$. $C_{30}H_{39}F_2N_4O_7$. Calculated, *m*/*z*: 605.2787.

Diastereomer 6B. Yield 16.1 mg (34%), white amorphous solid, $[\alpha]_D^{20}$ –27.6° (*c* 0.6, MeOH). ^{1}H NMR spectrum (CD_3OD), δ, ppm (*J*, Hz): 8.67 (1H, d, *J* = 7.2, NH); 7.90 (1H, s, H Ar); 7.06–6.94 (2H, m, H Ar); 6.81 (1H, tt, *J* = 8.8, *J* = 2.3, H Ar); 4.90–4.86 (1H, m, CH); 4.83–4.79 (1H, m, CH); 4.54 (2H, s, CH_2OH); 3.87 (1H, d, *J* = 3.4, CHOH); 2.59–2.42 (1H, m, CH_2CH); 2.27–2.15 (1H, m, CH_2CH); 2.15–2.05 (1H, m, $CH(CH_3)_2$); 1.98–1.76 (3H, m, CH_2CH, CH_2); 1.46–1.12 (12H, m, $C(CH_3)_3$, $2CH_2$); 0.99 (3H, d, *J* = 6.9, CH_3CH); 0.89–0.69 (3H, m, CH_3CH). ^{13}C NMR spectrum (CD_3OD), δ, ppm (*J*, Hz): 174.4; 173.4; 164.4 (dd, *J* = 248.0, *J* = 12.6); 156.3; 153.0; 143.0 (t, *J* = 8.4); 137.6; 137.0; 136.3; 125.9; 108.7–108.3 (m); 102.2 (t,



J = 26.0); 75.5; 73.8; 59.3; 55.3; 51.3; 38.1; 33.0; 31.6; 29.6; 25.6; 21.5; 19.9; 18.2; 14.7. ^{1}H NMR spectrum (DMSO-d_6), δ, ppm (*J*, Hz): 8.67 (1H, d, *J* = 7.3, NH); 8.08 (1H, s, H oxazole); 7.89 (1H, d, *J* = 2.2, ArCOH); 7.55 (1H, d, *J* = 7.7, NH); 7.14 (1H, tt, *J* = 9.0, *J* = 2.4, H Ar); 7.02–6.97 (1H, m, H Ar); 5.58 (1H, d, *J* = 5.7, $(CH_3)_2CCHO\underline{H}$); 5.34 (1H, t, *J* = 5.7, $CH_2O\underline{H}$); 4.82 (1H, ddd, *J* = 7.9, *J* = 5.0, *J* = 2.8, $C\underline{H}CH_2$); 4.68 (1H, d, *J* = 7.3, $(CH_3)_3C\underline{H}$); 4.40 (1H, d, *J* = 5.7, $C\underline{H}_2OH$); 3.70 (1H, dd, *J* = 5.7, *J* = 3.3, $C\underline{H}OH$); 2.41–2.35 (1H, m, $C\underline{H}_2COH$), 2.20–2.12 (1H, m, $C\underline{H}_2CH$); 1.99 (1H, sept d, *J* = 6.9, *J* = 3.3, $C\underline{H}(CH_3)_2$); 1.78–1.71 (1H, m, $C\underline{H}_2COH$); 1.71–1.62 (2H, m, $C\underline{H}_2CH$, CH_2); 1.14–1.05 (10H, m, $C(CH_3)_3$, CH_2); 0.89 (3H, d, *J* = 6.9, $C\underline{H}_3CH$); 0.72 (3H, d, *J* = 6.9, $C\underline{H}_3CH$), 0.66–0.58 (1H, m, CH_2). ^{13}C NMR spectrum (DMSO-d_6), δ, ppm (*J*, Hz): 172.7; 172.3; 162.3 (dd, *J* = 248.0, *J* = 13.2); 161.6; 155.1; 153.8; 151.5 (t, *J* = 8.4); 141.7; 124.1; 107.5–107.2 (m); 102.9 (t, *J* = 26.0); 74.8; 73.7; 58.5; 54.8; 50.3; 38.1; 33.1; 31.2; 29.4; 26.2; 21.3; 19.7; 19.1; 15.7. Found, *m*/*z*: 605.2792 $[M+H]^+$. $C_{30}H_{39}F_2N_4O_7$. Calculated, *m*/*z*: 605.2787.

Isothermal titration calorimetry. A stock solution of tubulin (5.0 mg/ml, Cytoskeleton Inc., Denver (USA)) was dissolved in the ice-cold 20 mM phosphate (NaPi) 0.1 mM GTP buffer (pH 6.5) just before the experiment and used within 6 h. Prechilled RB3 (STMN4) protein prepared in the same buffer was added to tubulin just before the experiment. Tubulin:RB3 concentration ratio varied from 2:1 to 1:1. Protein sample was stored on ice before loading into the ITC sample cell.

The ITC experiments have been performed in a MicroCal iTC200 calorimeter. The ITC system cleanliness was checked by water-to-water titration before every protein–ligand titration experiment. The temperature of the ITC cell was set up and equilibrated at 10°C. Afterward 280 µl of ice-cold tubulin protein sample was loaded into the cell. ITC syringe was prewashed with the same buffer that was used for the protein and was filled with the ice-cold ligand sample. Next, the ITC titration syringe was put into the sample cell and left for 5–10 min for temperature equilibration.

The titration experiment consisted of 1 × 0.3 µl and 26 × 1.5 µl injections with 125 s spacing in-between and at a stirring rate of 600 rpm. ITC data were analyzed using the Origin 7 SR4 program. The first data point (with the small injection volume) was always removed prior the data fitting. "One Set of Sites" fitting model was applied.

This work was funded by ERDF (project No. 1.1.1.1/16/A/281 "Simplified Analogues of Diazonamide A as Anticancer Agents").

We thank Prof. K. Jaudzems for assistance with NMR experiments and the ITC assay.

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