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Syntheses and cytotoxicity of syringolin B-based proteasome inhibitors

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Dedicated to Professor Gilbert Stork in honor of his 90th birthday, and in appreciation of his many decades of contributions to good chemistry and good humor

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ABSTRACT

The concise and modular total synthesis of the bacterial natural product and irreversible proteasome inhibitor syringolin B has been achieved. This synthesis has enabled the ready preparation of three diverse, structurally modified syringolin derivatives. The actions of these compounds in inhibiting the proliferation of neuroblastoma cell lines was evaluated, and significant enhancements in potency compared to the natural product were realized.

1. Introduction

The inhibition of protein degradation via the ubiquitin-proteasome system has recently been recognized as a useful mode of action for novel drugs.[1] One such FDA-approved drug, a boronic acid peptide analog called bortezomib, is used in the treatment of refractory multiple myeloma and mantle cell lymphoma. However, it has significant side effects (such as neuropathy), not all patients respond to it, and many responders eventually develop resistance.[2] Consequently, second-generation proteasome inhibitors are being actively sought, one approach being based on natural products. Many such compounds have been discovered, almost all inactivating the proteasome by reacting with it covalently. Two have entered clinical trials against multiple myeloma: a Phase 1 study of salinosporamide A is complete, and a Phase 2 study of carfilzomib, a modified version of two natural products, eponemycin and epoxomicin, achieved a significant response.[3]

This work focuses on a family of bacterial 12-membered lactam proteasome inhibitors called the syrbactins (Fig. 1). They include the syringolins,[4] glidobactins,[5] and cepafungins,[6] all of which express at least some anticancer activity. Their mode of action is via the conjugate addition of a proteasome hydroxyl group to the α,β-unsaturated amide. Among the natural proteasome inhibitors, the syrbactins provide an especially attractive starting point for therapeutic development because they can be synthesized in a relatively concise fashion. In addition to a classical synthesis of glidobactin A,[7] three total syntheses of syringolin A have been recently reported,[8] including our own;[9] Kaiser's lab and ours have also prepared syringolin B. Considerable follow-on work on the biological properties of syringolin derivatives has emerged from Kaiser and Bachmann.[10] A specific feature sought in our studies was a modular synthetic approach with potential for the preparation of structural variants by the substitution of modules, the concept of diversity-oriented synthesis.[11] Syringolin B is an appealing platform for a synthesis-based program toward novel

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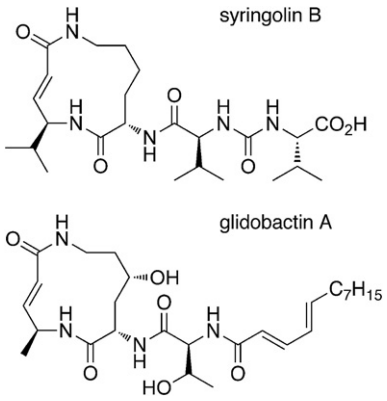


Fig. 1. Two representative syrbactin proteasome inhibitors.

proteasome inhibitors because a large portion of its structure is based on lysine, and many lysine analogs can be readily accessed. Here, we describe in full detail the total synthesis of syringolin B, the preparation of several structural variants, and their initial biological screening.

2. Results

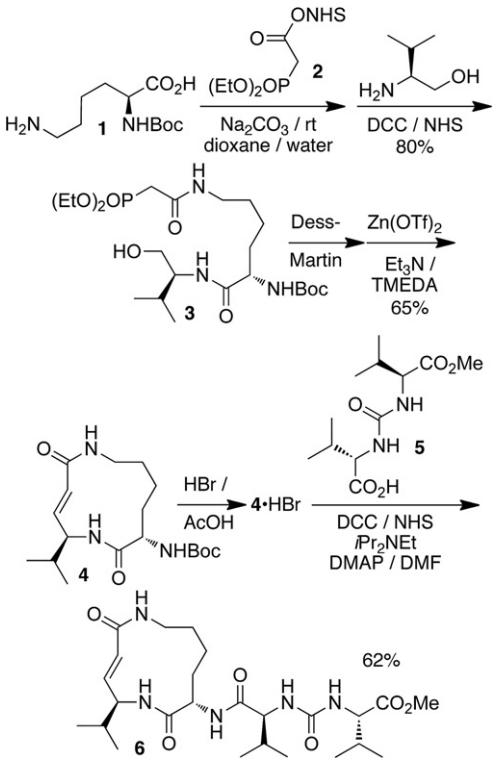
2.1. Total synthesis

The key to our approach to the syrbactins was the creation of the α,β-unsaturated 12-membered macrolactam via an intramolecular Horner–Wadsworth–Emmons (HWE) reaction. This reaction has strong precedents for the formation of large rings in high efficiency, including some larger than 30 atoms,[12] whereas other syrbactin syntheses have suffered from significant difficulties in closure of the large ring (15–49% yields).

The synthesis of syringolin B (Scheme 1) involves seven steps from the commercial Boc-Lys-OH **1**. The precursor of macrolactam **4**, the phosphono-alcohol **3**, was prepared in 80% yield for two steps. The first involves acylation using *N*-hydroxysuccinimide active ester **2**; the resulting lysine ε-phosphono-acetamide was coupled with L-valinol using *N*-hydroxysuccinimide (NHS) and dicyclohexylcarbodiimide (DCC) in dichloromethane. Phosphono-alcohol **3** was oxidized to the aldehyde under optimized conditions with 1.1 equiv of Dess–Martin periodinane in dichloromethane. This reaction proceeds with good yield and purity, as judged by NMR, but the crude aldehyde was used directly to minimize the possibility of enolization. These conditions were superior to alternatives examined, including the Swern modification of the Moffatt oxidation.

A few different conditions were examined for the HWE reaction. Sodium bis(trimethylsilyl)amide (NaHMDS) in THF afforded only decomposition products. The Roush–Masamune LiCl/DBU conditions gave no reaction. Treatment of the aldehyde with potassium carbonate and 18-crown-6[13] afforded the macrolactam as shown by NMR, but excess crown ether made its isolation impractical. Very mild HWE conditions consisting of zinc triflate, tetramethylethylenediamine (TMEDA), and triethylamine in THF at room temperature were developed by Schauer and Helquist for the preparation of α,β-unsaturated amides with high *E* selectivity.[14] When applied at high dilution over 20 h, macrolactam **4** was obtained in 65% yield from the phosphono-alcohol. Deprotection of the Boc group in **4** was performed with 33% HBr in acetic acid to afford its hydrobromide in essentially quantitative yield after 45 min at room temperature.

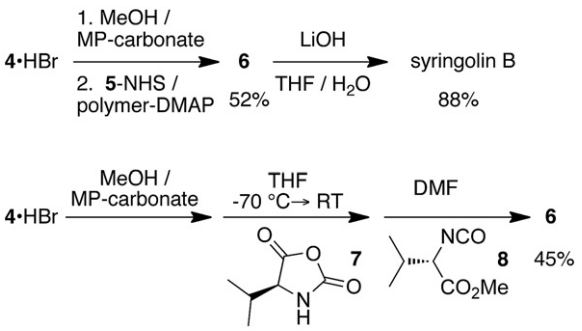
A number of tactics were examined for addition of the bis-valine urea side chain, the best two of which will be discussed.



Scheme 1. Synthesis of syringolin B methyl ester.

The first approach using a pre-formed side chain parallels the route used by Kaiser for the preparation of syringolin A.[8] Ester-acid **5** was prepared as described in their work. Early experiments showed that aqueous work-ups of these late-stage intermediates must be avoided due to their high polarity. The hydrobromide was neutralized in situ using diisopropylethylamine (DIEA) and treated with DCC, NHS, 3 equiv of acid **5**, and a catalytic amount of dimethylaminopyridine (DMAP) in DMF. The crude NMR spectrum showed the desired ester contaminated by DIEA hydrobromide, requiring chromatography to obtain compound **6** in 62% yield. It is notable that no stereoisomerization of the side chain fragment was observed even though the bis-valine urea was activated at its *C*-terminus. The side-chain coupling conditions of Kaiser, PyBOP (benzotriazol-1-yl-oxytripyrrolidino-phosphonium hexafluorophosphate), 1-hydroxy-7-azabenzotriazole (HOAt), and DIEA in DMF-dichloromethane, were inferior in this instance. The use of *p*-nitrophenol as activator instead NHS or carbonyl diimidazole as coupling agent instead of DCC also gave low yields. Pre-forming and recrystallizing the NHS active ester of **5** was also inferior.

A superior method for side-chain addition (Scheme 2) involved neutralizing the hydrobromide with a solid-phase base, MP-carbonate (methylpolystyrene-triethylammonium carbonate).[15] This is a macroporous polystyrene anion-exchange resin that is a resin-bound tetraalkylammonium carbonate equivalent. MP-Carbonate may be used as a general base to quench reactions, neutralize amine salts, or to scavenge a variety of acidic molecules, such as carboxylic acids or acidic phenols. When it was used to neutralize **4**·HBr in methanol and the free base was coupled to the NHS active ester of **5** in the presence of polymer-bound DMAP, **6** was obtained cleanly in 52% yield. Methyl ester **6** was subjected to hydrolysis using lithium hydroxide in THF/water to obtain syringolin B in very good yield, 88%. The less convenient conditions of Kaiser, $AlCl_3$ in methyl ethyl sulfide, gave syringolin B in 95% yield.



Scheme 2. Second-generation total syntheses of syringolin B.

These relatively modest results for what is essentially just a peptide coupling suggested that the steric environment of the macrolactam amine impedes reaction. We therefore sought activated amino acid derivatives that would present very small steric demands. Amino acid *N*-carboxyanhydrides (NCAs) are compact molecules with good acylation activity that are recognized for their ring-opening polymerization reactions to yield amino acid homopolymers.[16] Their ability to undergo single acylation reactions in organic solvents is less well-known. Using such reagents, methyl ester **6** could be prepared from **4** in a one-pot process without the need for side chain pre-synthesis. The macrolactam hydrobromide **4** was converted to the free base using MP-carbonate, followed by the addition of L-valine-NCA **7**. Without isolation, the resulting amine was treated with the commercial isocyanate **8** to give methyl ester **6** in moderate yield (though comparable to the addition of the preformed side chain). The yield was halved when dichloromethane rather than THF was the solvent for the acylation. This NCA route for adding the side chain is structurally versatile, streamlined, and quite atom-economical, with the only by-product being CO_2.

The NMR data for the synthetic syringolin B resulting from our syntheses was compared to literature data. Despite the fact that an authentic sample was unavailable for direct comparison, there is no doubt that the natural material was obtained. The overall yield for our best route is 31%.

2.2. Analog synthesis

Our initial consideration of syringolin B relatives to be targeted was influenced by the previous work of Kaiser on a molecule they call SylA-LIP (**9**) (Fig. 2). It is the most potent proteasome inactivator among the synthetic syringolins so far reported.[10] It presumably was designed to benefit from a side chain that resembles that of glidobactin A, the most potent of the natural syrbactins both in terms of proteasome inhibition and anticancer activity in cell-based assays.[10] Our first analog could be called SylB-LIP, though it has minor side chain variations from SylA-LIP. The one-pot method for side chain addition from our syringolin B total synthesis was emulated (Scheme 3). Neutralization of the macrolactam hydrobromide, acylation with the isoleucine-NCA, and urea formation with dodecylisocyanate led to compound **10.**

More interesting syringolin B relatives would modify the macrolactam core that is known to be the site of its reaction with the

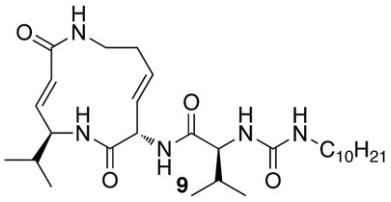
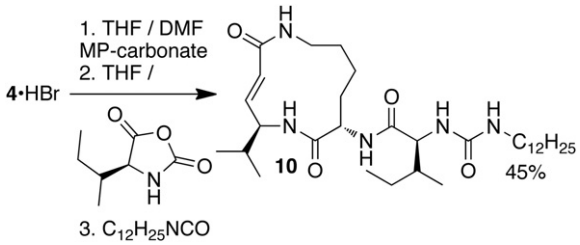
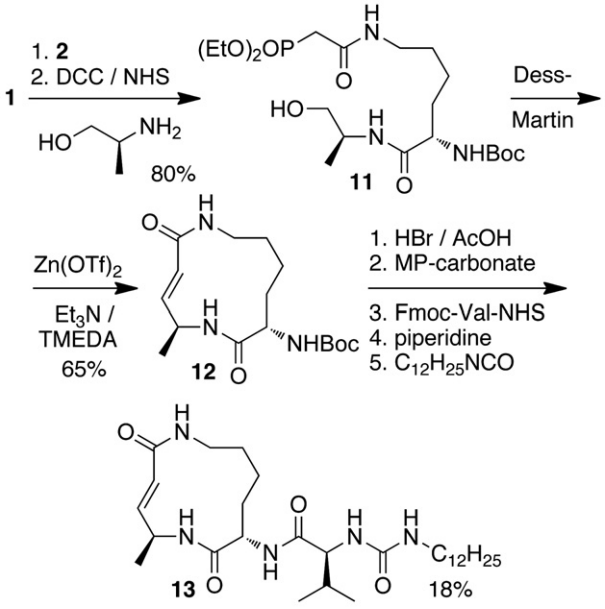


Fig. 2. Kaiser's syringolin A analog SylA-LIP.



Scheme 3. Synthesis of the syringolin analog SylB-LIP.

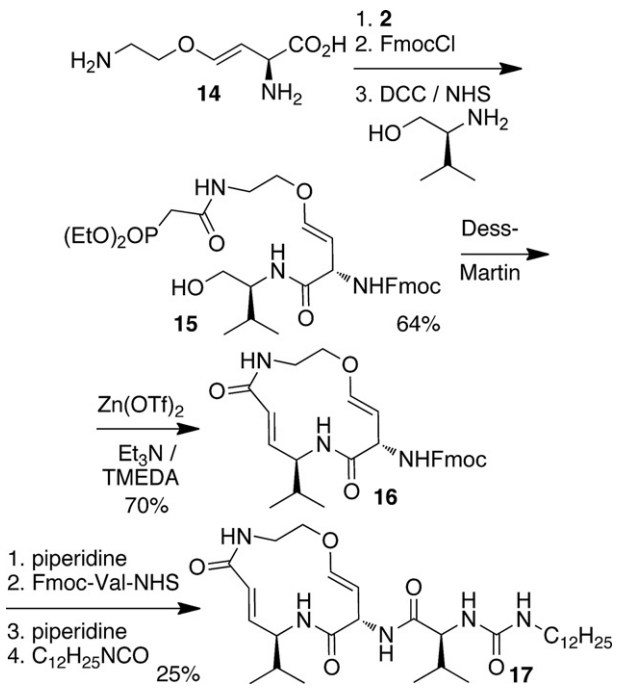
proteasome, exploiting our ability to use variant modules in our modular total synthesis. The isopropyl group was first changed to a methyl group to give a glidobactin-like macrolactam (Scheme 4). Following essentially the same route as earlier, L-alaninol was substituted for L-valinol. Phosphono-alcohol **11** was obtained in very good yield, and the HWE macrocyclization proceeded well. A new strategy was then used for the introduction of the side chain. Macrolactam **12** was deprotected as before and neutralized with MP-carbonate. The NHS active ester of Fmoc-valine was then coupled to the free amine. The intermediate was deprotected and the amine was treated with dodecylisocyanate to afford the desired product **13** in 18% yield over five steps (with purification of a single intermediate). By analogy to SylA-LIP, **13** is essentially dGlbA-LIP (deoxy-glidobactin A with the lipophilic side chain).



Scheme 4. Synthesis of the syringolin analog dGlbA-LIP.

The synthesis of our final syringolin B relative exploits an exotic lysine analog. Aminoethoxyvinylglycine (AVG) is a plant growth regulator that affects ethylene biosynthesis and plant physiology that is related to the phytohormone ethylene.[17] While the β,γ-unsaturated acid of AVG might be thought to make it more prone toward racemization and alkene isomerization, and its vinyl ether could make it sensitive to acid-based hydrolysis, the conjugation of the vinyl ether may also encourage the alkene to remain at the β,γ-position. A sample of **14** was obtained from a commercial source and converted to the vinyl ether/phosphono-alcohol **15** in three steps and decent yield (Scheme 5). It was submitted to our two-step oxidation/HWE macrocyclization procedure to produce the 13-membered vinyl ether macrolactam **16** in an excellent 70% yield. The side chain was completed using methods from the synthesis of

13 to afford **17** in 25% yield over four steps. This analog is essentially SylA-LIP with the addition of a single oxygen atom.



Scheme 5. Synthesis of the syringolin analog oxa-SylA-LIP.

2.3. Biological testing

The effects of these synthetic syringolins on the proliferation of the SK-N-AS and MYCN-2 neuroblastoma cell lines were examined in MTS cytotoxicity assays. The data are given graphically in Fig. 3. Compounds **10** and **13** have IC50s of ca. 1 μM, significant improvements upon the >20 μM for syringolin A[4] and comparable to the 3.2 μM reported for SylA-LIP (in a different cell line, SK-N-SH).[10] The most potent analog is **17**, with an IC50 of ca. 0.4 μM.

3. Discussion

The total synthesis of syringolin B described here is brief, efficient, versatile, and amenable to diversity-oriented synthesis. This versatility was demonstrated through the preparation of three syringolin B relatives with changes in the side chain and two of the three sub-segments of the macrolactam. The preparation of these analogs revealed areas for further research on syrbactin synthesis. More reliable approaches to side-chain attachment are surely needed, and better methods to permit the formation of the macrolactam with the small alkyl groups that provide better biological activity (vide infra) are also needed. These subjects will be pursued as structure–activity relationships are defined.

The results of testing these new analogs add to the understanding of structural features that affect the biological activity of the syrbactins. More information, in particular their intrinsic rates of reaction with each of the three proteasome catalytic subunits, is needed before their activities can be interpreted on a molecular basis. However, at least three structural considerations influenced the design of these analogs. First, the greater potency of glidobactin A among the syrbactins, its lipophilic side chain, and the side chain used by Clerc et al.[10] in SylA-LIP prompted our selection of a very similar side chain design. Second, it is apparent in the crystal structures of syringolin A and glidobactin A bound to the proteasome[18] that the nucleophilic Thr1 hydroxyl that adds to the α,β-unsaturated amide approaches the ring from the same face as the isopropyl or methyl groups, respectively. Because glidobactin A is more potent than syringolin A, it was thought that a smaller alkyl substituent adjacent to the site of nucleophilic attack could facilitate conjugate addition. Of course, that substituent is not the only difference between them.

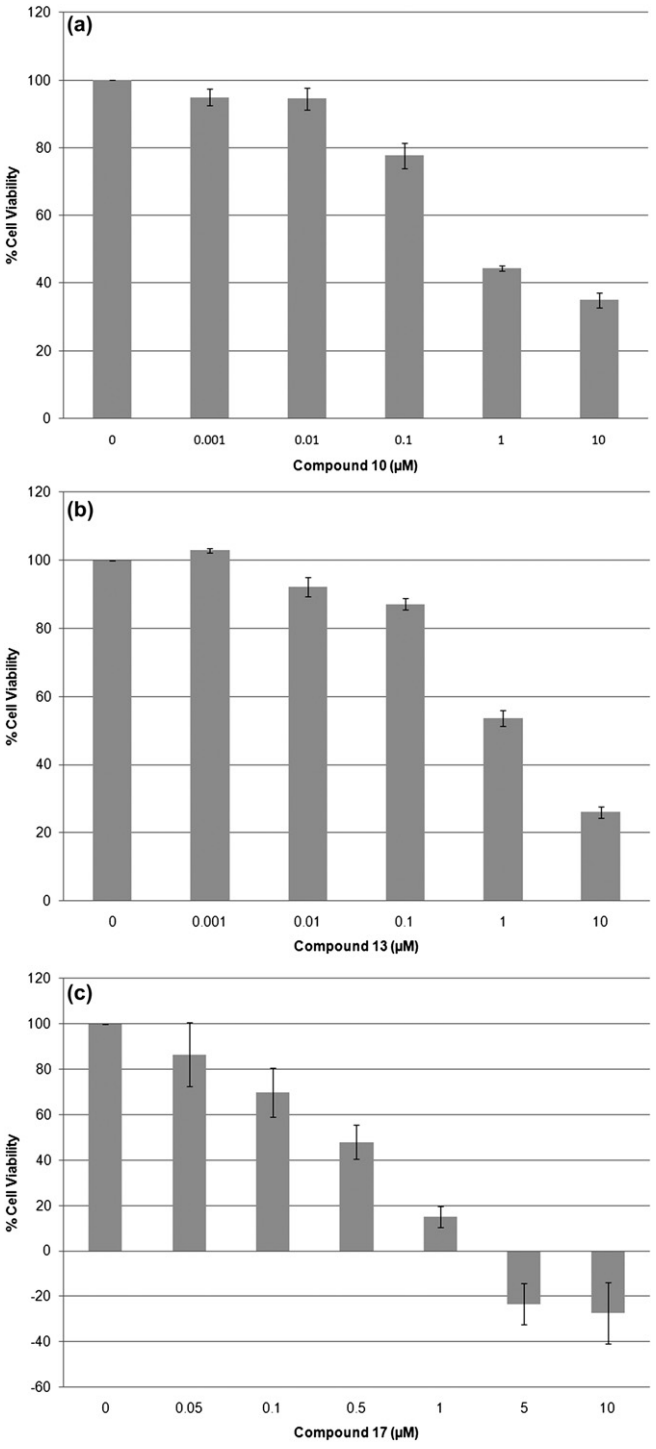


Fig. 3. Syrbactin analogs inhibit the proliferation of human neuroblastoma (NB) cells. NB is a cancer of childhood and establishes in the sympathetic nervous system. The human NB cell lines SK-N-AS (*A*, *B*) and MYCN-2 (*C*) were treated individually over a period of 48 h with compounds **10** and **13** (*A*, *B*) or **17** (*C*) at various concentrations (0–10 μM). The viability of cells was determined by MTS assay as outlined in the Experimental section. Data normalized to controls (untreated cells) represent the mean of two (*A*, *B*) or three (*C*) independent experiments, and each experiment was performed in triplicate wells; error bars, ±SD.

Another difference is the additional alkene within the syringolin A macrolactam. We thought the second alkene would increase strain, potentially making the α,β-unsaturated amide more reactive to nucleophilic addition through relief of this strain. Force field calculations (MMFF94) were performed on model macrolactams for syringolin A, glidobactin A, and syringolin B with formyl and methyl groups as surrogate side chains. All adopt similar conformations, with the planes of the amides and the alkenes perpendicular to the nominal ring plane, as is the norm for macrocyclic alkenes (Fig. 4). The glidobactin A and syringolin B cores have comparable strain energies, while the syringolin A core has ca. 13 kcal/mol higher energy. That analysis applied directly to **10** and **13**, but not to **17**, since it has an additional atom in the ring that could relieve some of the strain. However, MMFF94 calculations show that there is little difference between the strain of its core and the syringolin A core. AVG could therefore prove very attractive as a pre-assembled β,γ-unsaturated lysine that makes for shorter syntheses of novel syrbactins with cores comparable to syringolin A. Alternatively, Clerc et al.[10] proposed that the second unsaturation of syringolin A promotes a different conformation than syringolin B or glidobactin A that is more apt for reaction with the proteasome.

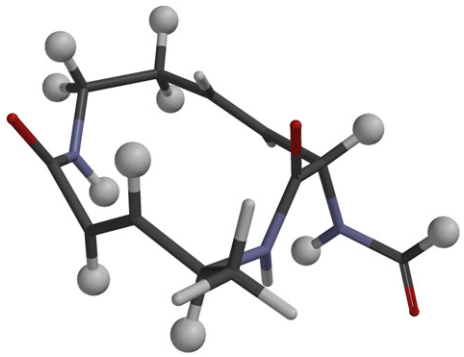


Fig. 4. Force field minimized conformer of a syringolin A model macrolactam.

In summary, the preparation of syringolin B and its congeners using diversity-oriented synthesis has provided structure–activity relationships of the syrbactins that can be used to develop next-generation proteasome inhibitors and potential anticancer agents. Further biological studies on molecules in this family are reported separately.[19]

4. Experimental section

4.1. General

All melting points were measured on a Büchi Melting Point B-545 and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on Varian Inova 300 (300 MHz and 75 MHz, respectively) or Varian Inova 400 (400 MHz and 100 MHz, respectively) as noted, are internally referenced to residual solvent signals, and are expressed in parts per million (ppm). IR spectra were recorded on a Perkin–Elmer Spectrum One FT-IR spectrometer using the ATR accessory. Mass spectra were obtained in the UCR Analytical Chemistry Instrument Facility with an Agilent G3250AA LC/MSD TOF. The purity of all final compounds was established by HPLC/MS (MeOH/C18). Intermediates **2**–**4** and **6** in the synthesis of syringolin B were reported in the Supplementary data of our earlier paper.[9]

*4.1.1. (2S,3R)-2-(3-Dodecylureido)-N-((5S,8S,E)-5-isopropyl-2,7-dioxo-1,6-diazacyclododec-3-en-8-yl)-3-methylpentanamide (**10**).* Hydrobromic acid in acetic acid (33%, 0.6 mL) was added to a solution of macrolactam **4** (22 mg, 0.071 mmol) in acetic acid (0.2 mL) at room temperature. The reaction mixture was stirred for 45 min and 6 mL of ethyl acetate was added. The resulting orange suspension was stirred at 0–5 °C for 30 min, the residue was collected by centrifugation, and the product was dried in vacuo. This solid HBr salt (20 mg, 0.060 mmol, 100%) was redissolved in a 1:1 mixture of tetrahydrofuran/dimethylformamide (0.4 mL) and MP-carbonate resin (72.3 mg, 3.10 mmol/g, 0.242 mmol) was added at 0 °C. The reaction mixture was stirred at room temperature for 30 min and then cooled to −70 °C (*S*)-4-*s*-butyloxazolidine-2,5-dione (L-isoleucine *N*-carboxyanhydride, 10 mg, 0.063 mmol) in THF (0.1 mL) was added drop-wise at −70 °C. The resulting mixture was stirred at that temperature for 12 h, was allowed to warm to room temperature, and was stirred for 8 h. Dodecylisocyanate (13.4 mg, 0.0634 mmol) was added at room temperature and the reaction mixture was stirred for 15 h. It was concentrated and the residue was purified by chromatography (SiO_2, methanol/dichloromethane gradient, 0:1 to 1:9) to give the title compound (15 mg, 45% for three steps). ^{1}H NMR (DMSO-d_6): δ 8.25 (dd, *J*=16.8, 8.2 Hz, 1H), 7.34 (d, *J*=8.2 Hz, 1H), 6.76 (dd, *J*=15.3, 4.6 Hz, 1H), 6.21 (d, *J*=15.3 Hz, 1H), 5.89 (m, 1H), 4.53–4.48 (m, 1H), 4.12–4.02 (m, 3H), 2.93–2.82 (m, 4H), 2.03–1.99 (m, 2H), 1.76–1.73 (m, 3H), 1.27 (br s, 25H), 1.12 (t, *J*=6.8 Hz, 6H), 0.99–0.85 (m, 9H). Mp: 234–237 °C. HRMS calcd for $C_{32}H_{60}N_5O_4$ $[M+H]^+$ 578.4645, found 578.4593. IR (neat): 3336, 2955, 2920, 2848, 1612, 1571, 1465 cm^{-1} $[\alpha]_D^{25}$ −10.7 (*c* 0.2, MeOH). This compound was too insoluble to obtain a ^{13}C NMR spectrum.

*4.1.2. tert-Butyl ((S)-6-(2-(diethoxyphosphoryl)acetamido)-1-(((S)-1-hydroxypropan-2-yl)amino)-1-oxohexan-2-yl)carbamate (**11**).* Boc-Lys-OH (500 mg, 2.03 mmol) was dissolved in dioxane/water (1:1, 5 mL), and the solution was cooled to 0 °C. Sodium carbonate (215 mg, 2.03 mmol) was added and the solution was stirred at 0 °C for 30 min. A solution of **2** (595 mg, 2.30 mmol) in 1 mL of dioxane was added drop-wise. The mixture was stirred for 1 h at 0 °C and 15 h at room temperature. Organic impurities were removed by washing with ethyl acetate (20 mL). The aqueous phase was acidified to pH 2 with 1 N HCl and extracted with ethyl acetate (4×15 mL). The combined organic extracts were dried and concentrated to afford 780 mg of colorless oil that was used in the next step without further purification.

To a stirring suspension of crude *N*′-diethylphosphonacetyl-*N*-Boc-Lys-OH (780 mg, 1.84 mmol) in dry dichloromethane (20 mL), *N*-hydroxysuccinimide (211 mg, 1.84 mmol), and *N*,*N*′-dicyclohexylcarbodiimide (398 mg, 1.93 mmol) were added at 0 °C, followed by L-alaninol (138 mg, 1.84 mmol). The mixture was stirred for 20 h at room temperature and *N*,*N*′-dicyclohexylurea was filtered off. The filtrate was concentrated to yield a yellowish oil that was purified by chromatography (SiO_2, acetone/dichloromethane gradient, 1:1 to 9:1) to afford the title compound as a colorless oil (700 mg, 80% for two steps). ^{1}H NMR (CD_3OD): δ 4.15 (quint, *J*=7.5 Hz, 4H), 3.94 (sext, *J*=5.7 Hz, 2H), 3.48–3.45 (m, 2H), 3.19 (t, *J*=6.9 Hz, 2H), 2.94 (d, *J*=21.9 Hz, 2H), 1.78–1.69 (m, 1H), 1.57–1.51 (m, 2H), 1.44 (s, 9H), 1.33 (t, *J*=6.9 Hz, 6H), 1.16 (d, *J*=6.9 Hz, 3H). ^{13}C NMR (CD_3OD): δ 172.3, 164.3, 155.6, 79.3, 65.5, 62.6, 62.5, 54.4, 47.3, 39.2, 35.1 (d, *J*=131 Hz), 32.2, 28.1, 22.4, 16.6, 16.1. IR (neat): 3262, 2988, 1712, 1687, 1230 cm^{-1}. HRMS calcd for $C_{20}H_{40}N_3O_8P$ $[M+H]^+$ 482.2587, found 482.2594. $[\alpha]_D^{25}$ −8.5 (*c* 2.0, MeOH).

*4.1.3. tert-Butyl ((5S,8S,9E)-5-methyl-2,7-dioxo-1,6-diazacyclododeca-3,-en-8-yl)carbamate (**12**).* To a stirring suspension of phosphono-alcohol **11** (110 mg, 0.224 mmol) in dry dichloromethane (2 mL) was added Dess–Martin periodinane (109 mg, 0.257 mmol) at room temperature for 30 min. The reaction mixture was diluted with ethyl acetate (10 mL) and 1 mL of a 1:1 mixture of sat $NaHCO_3$ and 2% sodium thiosulfate. The mixture was stirred vigorously for 5 min (until the organic phase was clear) and the phases were separated. The organic phase was dried over sodium sulfate, filtered, and concentrated in vacuo to yield the aldehyde that

was used without purification. Tetramethylethylenediamine (0.026 mL, 0.18 mmol) and triethylamine (0.080 mL, 0.59 mmol) were added to a suspension of $Zn(OTf)_2$ (149 mg, 0.332 mmol) in THF (70 mL) at room temperature. After 15 min the phosphono-aldehyde (75 mg, 0.15 mmol) in THF (10 mL) was added drop-wise over 60 min. The resulting mixture was stirred for 15 h at room temperature and concentrated to 10 mL. The residue was diluted with ethyl acetate (100 mL) and washed with brine (70 mL) and 1% HCl (70 mL). The organic phase was dried over sodium sulfate and concentrated to afford a clear oil that was purified by chromatography (SiO_2, acetone/dichloromethane gradient, 4:6 to 8:2) to afford the title compound as a white solid (48 mg, 65% for two steps). Mp: 262.3–263.7 °C ^{1}H NMR (CD_3OD): δ 6.95 (dd, J=15.4, 4.9 Hz, 1H), 6.35 (d, J=15.4 Hz, 1H), 4.30–4.18 (m, 1H), 3.18–3.15 (m, 1H), 3.21 (t, J=5.7 Hz, 2H), 1.86–1.78 (m, 4H), 1.67–1.64 (m, 2H), 1.46 (s, 9H), 1.22 (d, J=6.7 Hz, 3H). ^{13}C NMR (CD_3OD): δ 172.4, 168.5, 156.1, 148.2, 118.2, 79.4, 53.4, 46.3, 38.5, 30.2, 30.0, 17.5, 17.4. IR (neat): 3279, 2931, 2857, 1650, 1544, 1415 cm^{-1}. HRMS calcd for $C_{16}H_{27}N_3NaO_4$ $[M+Na]^+$ 348.1894, found 348.1909. $[\alpha]_D^{25}$ −39.3 (*c* 0.9, MeOH).

4.1.4. (9H-Fluoren-9-yl)methyl (R)-3-methyl-1-((5S,8S,E)-5-methyl-2,7-dioxo-1,6-diazacyclododec-3-en-8-ylamino)-1-oxobutan-2-ylcarbamate. Hydrobromic acid in acetic acid (33%, 0.6 mL) was added to a solution of macrolactam **12** (27 mg, 0.061 mmol) in acetic acid (0.3 mL) at room temperature. The reaction mixture was stirred for 45 min and 6 mL of ethyl acetate was added. The resulting orange suspension was stirred at 0–5 °C for 30 min, the residue was collected by centrifugation, and the product was dried in vacuo. This solid HBr salt (23 mg, 0.082 mmol, 96%) was redissolved in dimethylformamide (0.5 mL) and MP-carbonate resin (167 mg, 3.10 mmol/g, 0.247 mmol) was added at 0 °C followed by Fmoc-Val-NHS (35 mg, 0.079 mmol). The resulting mixture was allowed to warm and stirred during 24 h at room temperature. The crude material was purified by chromatography (SiO_2, methanol/dichloromethane gradient, 0:1 to 1:9) to give a white solid (12.3 mg, 45%). Mp: 176–178 °C $[\alpha]_D^{25}$ −11.8 (*c* 0.8, MeOH). HRMS calcd for $C_{31}H_{39}N_4O_5$ $[M+H]^+$ 547.2915, found 547.2932. IR (neat): 3300, 2960, 1668, 1542, 1450, 1258 cm^{-1} ^{1}H NMR (CD_3OD): δ 7.80 (d, J=7.4 Hz, 2H), 7.70–7.59 (m, 2H), 7.35 (m, 4H), 7.01 (dd, J=15.4, 4.8 Hz, 1H), 6.38 (d, J=15.3 Hz, 1H), 4.68–4.46 (m, 2H), 4.37 (d, J=6.7 Hz, 1H), 4.21 (m, 2H), 4.01–3.85 (m, 1H), 3.49 (m, 1H), 3.09 (dd, J=14.7, 2.8 Hz, 1H), 1.85 (dd, J=11.2, 4.4 Hz, 1H), 1.58 (dd, J=10.8, 4.1 Hz, 4H), 1.48–1.25 (m, 4H), 1.17 (d, J=6.6 Hz, 3H), 0.99 (m, 6H). ^{13}C NMR (CD_3OD): δ 172.5, 168.1, 157.3, 148.1, 143.4, 141.1, 127.2, 126.7, 124.7, 119.3, 118.1, 66.6, 53.3, 52.4, 46.4, 38.8, 30.6, 30.5, 29.9, 29.6, 29.2, 18.5, 17.5, 17.4, 15.6.

*4.1.5. (S)-2-(3-Dodecylureido)-3-methyl-N-((5S,8S,E)-5-methyl-2,7-dioxo-1,6-diazacyclododec-3-en-8-yl)butanamide (**13**).* Piperidine (3 μL) was added to a solution of the above compound (10 mg, 0.017 mmol) in DMF (0.5 mL). The solution was stirred over 30 min and evaporated. The mixture was diluted with DMF (0.5 mL) and dodecylisocyanate (5 μL, 0.021 mmol) was added. The resulting mixture was stirred for 24 h at room temperature and concentrated in vacuo to afford a white solid that was purified by chromatography (SiO_2, methanol/dichloromethane gradient, 0:1 to 2:8) to afford a white solid (5 mg, 41%). Mp: 245–248 °C ^{1}H NMR (DMSO-d_6): δ 8.37 (d, J=7.8 Hz, 1H), 7.67 (d, J=6.6 Hz, 1H), 7.38–7.23 (m, 1H), 6.76 (dd, J=15.1, 4.4 Hz, 1H), 6.23 (d, J=15.1 Hz, 4H), 5.95 (d, J=8.3 Hz, 1H), 4.54–4.45 (m, 1H), 4.44–4.32 (m, 1H), 4.09–3.96 (m, 1H), 3.03–2.84 (m, 2H), 2.01–1.89 (m 1H), 1.67–1.60 (m, 2H), 1.57–1.47 (m, 4H), 1.23 (br s, 24H), 0.91–0.66 (m, 9H). HRMS calcd for $C_{29}H_{54}N_5O_4$ $[M+H]^+$ 536.4176, found 536.4130. IR (neat): 3302, 2932, 1691, 1654, 1517 cm^{-1} $[\alpha]_D^{25}$ −15.8 (*c* 0.2, MeOH). This compound was too insoluble to obtain a ^{13}C NMR spectrum.

*4.1.6. (9H-Fluoren-9-yl)methyl((S,E)-4-(2-(2-(diethoxyphosphoryl) acetamido)-1-)(((S)-1-hydroxy-3-methylbutan-2-yl)amino)-1-oxobut-3-en-2-yl)carbamate (**15**).* (*S,E*)-4-(2-Aminoethoxy)-2-amin obut-3-enoic acid (**14** hydrochloride, ca. 92% pure commercial product, 200.0 mg, 0.936 mmol) was dissolved in dioxane/water (1:1, 6 mL), and the solution was cooled to 0 °C. Sodium carbonate (198.7 mg, 1.870 mmol) was added and the solution was stirred at 0 °C for 30 min. A solution of **2** (366.2 mg, 1.250 mmol) in 0.5 mL of dioxane was added drop-wise. The mixture was stirred for 1 h at 0 °C and 15 h at room temperature. (Fluorenylmethyloxy)carbonyl chloride (388.1 mg, 1.5 mmol) was added at 0 °C and the mixture was stirred 1 h at 0 °C and 15 h at room temperature. Organic impurities were removed by washing with ethyl acetate (60 mL). The aqueous phase was acidified to pH 2 with 1 N HCl and extracted with ethyl acetate (4×50 mL). The combined organic extracts were dried and concentrated to afford 460 mg of yellowish oil. This material was used in the next step without further purification.

N,N′-Dicyclohexylcarbodiimide (185.4 mg, 0.90 mmol) was added to a solution (0 °C) of *N*-hydroxysuccinimide (103 mg, 0.90 mmol) and L-valinol (92.7 mg, 0.90 mmol) in THF (5 mL). The reaction was stirred for 15 h at room temperature, the precipitate (*N,N′*-dicyclohexylurea) was filtered off, and the filter cake was washed with 5 mL of THF. Ethyl acetate (10 mL) was added to the filtrate, which was stored for 15 min at 5 °C. Additional *N,N′*-dicyclohexylurea was filtered off, the filtrate was dried over sodium sulfate, filtered, and concentrated in vacuo to give 390 mg of the title compound (64%). ^{1}H NMR ($CDCl_3$): δ 7.75 (d, J=7.2 Hz, 2H), 7.62 (d, J=7.2 Hz, 2H), 7.33 (t, J=7.8 Hz, 2H), 7.28 (t, J=7.8 Hz, 2H), 6.65–6.60 (m, 1H), 4.89 (t, J=8.3 Hz, 1H), 4.60–4.58 (m, 1H), 4.36 (s, 2H), 4.17–4.11 (m, 8H), 3.86–3.79 (m, 2H), 3.70–3.58 (m, 3H), 2.90 (d, J=20.8 Hz, 2H), 2.01–1.97 (m, 1H), 1.36–1.31 (m, 6H), 0.98 (d, J=6.8 Hz, 3H), 0.95 (d, J=6.8 Hz, 3H). ^{13}C NMR ($CDCl_3$): δ 171.2, 164.7, 155.9, 151.2, 143.9, 141.3, 127.8, 127.2, 125.3, 120.0, 102.2, 68.9, 67.1, 63.3, 62.7, 57.3, 54.0, 47.2, 39.8, 35.2 (d, J=144 Hz,) 29.2, 19.6, 18.7, 16.3. IR (neat): 3297, 2962, 1718, 1655, 1534, 1240, 1025 cm^{-1}. HRMS calcd for $C_{32}H_{44}N_3O_9P$ $[M+H]^+$ 646.2893, found 646.2914. $[\alpha]_D^{25}$ +2.7 (*c* 1.6, MeOH).

*4.1.7. (9H-Fluoren-9-yl)methyl(6E,8S,11S,12E)-8-isopropyl-5-10-dioxo-1-oxa-4,9-diazacyclotrideca-6-12-dien-11-carbamate (**16**).* To a stirring solution of phosphono-alcohol **15** (300 mg, 0.465 mmol) in dry dichloromethane (5 mL) was added Dess-Martin periodinane (236 mg, 0.558 mmol) at room temperature for 30 min. The reaction mixture was diluted with dichloromethane (20 mL) and 3 mL of a 1:1 mixture of sat $NaHCO_3$ and 2% sodium thiosulfate. The mixture was stirred vigorously for 5 min (until the organic phase was clear) and the phases were separated. The organic phase was dried over sodium sulfate, filtered, and concentrated in vacuo to yield 220 mg of aldehyde that was used without purification in the next step.

Tetramethylethylenediamine (62 μL, 0.41 mmol) and triethylamine (0.192 mL, 1.37 mmol) were added to a suspension of $Zn(OTf)_2$ (347.4 mg, 0.750 mmol) in THF (150 mL) at room temperature. After 15 min the phosphono-aldehyde (220 mg, 0.341 mmol) in THF (20 mL) was added drop-wise over 2 h. The resulting mixture was stirred for 15 h at room temperature and concentrated to 10 mL. The residue was diluted with ethyl acetate (100 mL) and washed with brine (70 mL) and 1% HCl (70 mL). The organic phase was dried over sodium sulfate and concentrated to afford a clear oil that was purified by chromatography (SiO_2, acetone/dichloromethane gradient, 1:9 to 7:3) to afford the title compound as a white solid (70% for two steps). Mp: 165–168 °C. IR (neat): 3300, 2960, 1668, 1542, 1450, 1258 cm^{-1} ^{1}H NMR (CD_3OD): δ 8.01 (d, J=9.6 Hz, 1H), 7.79 (d, J=7.5 Hz, 2H), 7.66 (d, J=6.6 Hz, 2H), 7.40 (t, J=6.9 Hz, 2H), 7.28 (t, J=6.6 Hz, 2H), 6.85 (dd, J=15.3, 3.9 Hz, 1H), 6.72 (d, J=12.6 Hz, 1H), 6.57 (dd, J=15.6, 1.8 Hz, 1H), 4.68 (d, J=9.6 Hz, 1H), 4.39–4.32 (m, 2H), 4.28–4.11 (m, 2H), 4.10–3.92 (m, 2H), 3.88–3.62 (m, 2H), 3.59–3.48 (m, 1H), 1.93–1.85 (m, 1H), 1.03–0.90 (m, 6H). ^{13}C NMR (CD_3OD): δ 174.9, 170.3, 150.8, 146.7,

145.0, 142.4, 128.6, 128.0, 126.1, 121.9, 120.8, 105.8, 70.6, 67.9, 57.4, 55.6, 41.8, 33.0, 19.7, 19.4. HRMS calcd for $C_{28}H_{32}N_3O_5$ $[M+H]^+$ 490.2342, found 490.2325. $[\alpha]_D^{25}$ −23.7 (*c* 0.8, MeOH).

4.1.8. (9H-Fluoren-9-yl)methyl (S)-1-((6E,8S,11S,12E)-8-isopropyl-5,10-dioxo-1-oxa-4,9-diaza cyclotrideca-6,12-dien-11-ylamino)-3-methyl-1-oxobutan-2-ylcarbamate. Piperidine (3 μL) was added to a solution of macrolactam **16** (20 mg, 0.041 mmol) in DMF (0.5 mL). The resulting mixture was stirred at room temperature during 30 min and volatiles were evaporated. The residue was redissolved in DMF (0.5 mL) followed by the addition of Fmoc-Val-NHS (21.3 mg, 0.049 mmol) at 0 °C. The resulting mixture was allowed to warm and stirred during 18 h at room temperature. The crude material was purified by chromatography (methanol/dichloromethane gradient, 0:1 to 1:9) to give 14 mg (62%) of a white solid. Mp: 112–115 °C $[\alpha]_D^{25}$ −13.8 (*c* 0.7, MeOH). ^{13}C NMR (CD_3OD): δ 175.1, 168.1, 166.7, 157.3, 143.9, 143.5, 141.4, 127.6, 127.0, 125.0, 119.7, 66.7, 61.5, 60.3, 55.2, 43.3, 41.8, 35.7, 30.3, 26.3, 25.5, 25.0, 24.1, 19.3, 18.2, 17.3, 15.7. HRMS calcd for $C_{33}H_{40}N_4O_6$ $[M+H]^+$ 589.2947, found 589.2962. IR (neat): 3289, 2961, 1780, 1702, 1529, 1449, 1210 cm^{-1} ^{1}H NMR (CD_3OD): δ 8.33 (d, *J*=9.4 Hz, 1H), 7.80 (d, *J*=7.5 Hz, 2H), 7.73–7.62 (m, 2H), 7.35 (dt, *J*=25.5, 7.6 Hz, 4H), 6.95 (d, *J*=6.2 Hz, 2H), 6.13 (d, *J*=5.9 Hz, 1H), 5.53–5.48 (m, 1H), 5.31–5.18 (m, 1H), 4.58–4.47 (m, 1H), 4.37 (dd, *J*=6.5, 4.0 Hz, 2H), 4.30–4.18 (m, 2H), 4.06 (dd, *J*=10.7, 4.8 Hz, 2H), 4.00–3.85 (m, 1H), 2.12–1.95 (m, 1H), 1.91–1.77 (m, 1H), 1.09–0.83 (m, 12H).

*4.1.9. (S)-2-(3-Dodecylureido)-N-((6E,8S,11S,12E)-8-isopropyl-5–10-dioxo-1-oxa-4,9-diazacyclotrideca-6-12-dien-11-yl)-3-methylbutanamide (**17**).* Piperidine (3 μL) was added to a solution of the above compound (10 mg, 0.017 mmol) in DMF (0.5 mL) and stirred over 30 min and volatiles were evaporated. To the residue was added DMF (0.5 mL) and dodecylisocyanate (5 μL, 0.021 mmol). The resulting mixture was stirred at room temperature during 15 h and the crude was concentrated in vacuo to afford a white solid that was purified by chromatography (SiO_2, methanol/dichloromethane gradient, 0:1 to 2:8) to afford a white solid (4 mg, 41%). Mp: 168–171 °C. HRMS calcd for $C_{31}H_{55}N_5O_5$ $[M+H]^+$ 578.4281, found 578.4291. IR (neat): 3298, 2963, 2874, 1703, 1645, 1215 cm^{-1} ^{1}H NMR (CD_3OD): δ 7.80 (d, *J*=9.6 Hz, 1H), 6.92 (m, 2H), 6.30–5.90 (m, 2H), 5.17 (t, *J*=8.1 Hz, 1H), 4.72 (s, 1H), 4.26–3.77 (m, 2H), 3.61–3.43 (m, 2H), 3.08 (s, 2H), 2.94–2.83 (m, 1H), 2.28 (m, 1H), 2.07 (d, *J*=7.8 Hz, 1H), 1.65 (s, 1H), 1.53–1.18 (m, 22H), 0.94 (m, 15H). $[\alpha]_D^{25}$ −12.1 (*c* 0.5, MeOH). This compound was too insoluble to obtain a ^{13}C NMR spectrum.

4.2. Mammalian cell cultures and reagents

The human neuroblastoma (NB) cell line SK-N-AS was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The human NB cell line MYCN-2[20] is derived from line SH-EP and was kindly provided by Dr. J. Shohet (Texas Children's Hospital). Cell lines were maintained in RPMI 1640 medium (SK-N-AS) or DMEM medium (MYCN-2) from Biosource (Rockeville, MD, USA) containing 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA). SK-N-AS cells were supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL). All cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

4.3. Cytotoxicity assay

The CellTiter 96 Aqueous One solution Cell Proliferation Assay (2-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; MTS) (Promega, San Luis Obispo, CA, USA) was used to determine the viability of NB cells after 48 h treatment with compounds **10**, **13**, and **17** at indicated concentrations by measuring the absorbance at 490 nm using a bioassay plate reader and data expressed in percent (%) cell viability relative to control (untreated) cells. All experiments were performed in triplicate wells in two or three independent experiments. Error bars indicate standard deviations (SD).

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Supplementary data

^{1}H NMR spectra for **10**–**13** and **15**–**17**. Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2011.09.048. These data include MOL files and InChiKeys of the most important compounds described in this article.

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