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Succinamide derivatives of melampomagnolide B and their anti-cancer activities

Venumadhav Janganati^a, Jessica Ponder^c, Shraddha Thakkar^d, Craig T. Jordan^b, and Peter A. Crooks^{a,*}

^aDepartment of Pharmaceutical Sciences, College of Pharmacy, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA

^bDivision of Hematology, University of Colorado, Aurora, CO 80045, USA

^cDepartment of Toxicology, University of Colorado, Aurora, CO 80045, USA

^dNational Center for Toxicological Research, Jefferson, AR 72079 USA

Abstract

A series of succinamide derivatives of melampomagnolide B have been synthesized by coupling MMB monosuccinate (**2**) with various heterocyclic amines to afford compounds **3a–3l**. MMB monosuccinate was also reacted with terminal diaminoalkanes to afford dimeric succinamido analogs of MMB (**4a–4h**). These succinamide analogs of MMB were evaluated for their anti-cancer activity against a panel of sixty human cancer cell lines. Analogs **3d–3i** and dimers **4f–4g** exhibited promising anti-cancer activity with GI₅₀ values ranging from 0.28–33.5 μ M against most of the cell lines in the panel. The dimeric analogs **4f** and **4g** were identified as lead compounds with GI₅₀ values in the nanomolar range (GI₅₀ = 280–980 nM) against several cell lines in the panel; i.e. leukemia cell lines CCRF-CEM, HL-60(TB), K-562, MOLT-4, RPMI-8226 and SR; and solid tumor cell lines NCI-H522 (non-small cell lung cancer), SW-620 and HCT-116 (colon cancer), LOX IMVI (melanoma), RXF 393 (renal cancer), and MCF7, BT-549 and MDA-MB-468 (breast cancer). Succinamide analogs **3a**, **3c–3l** and **4b–4h** were also evaluated for their apoptotic activity against M9-ENL1 acute myelogenous leukemia cells; compounds **3h–3j** and **4g** were equipotent with parthenolide, exhibiting LC₅₀ values in the range 4.1–8.1 μ M. Molecular docking studies indicate that these molecules interact covalently with the highly conserved Cys-46 residue of the N-terminal lobe (1–109) of human IKK β to inhibit the NF κ B transcription factor complex, resulting in down-regulation of anti-apoptotic genes under NF κ B control.

Graphical abstract

*Corresponding author. Tel.: +1 501 686 6495; fax: +1 501 686 6057. pacrooks@uams.edu.

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Keywords

Parthenolide; Melampomagnolide B; Anti-cancer activity; Succinamide derivatives; IKK β inhibition

1. Introduction

Cancer has been and continues to be one of the major areas of focus for drug discovery. Yet, despite the advent of improved anticancer agents over the last thirty years, currently available chemotherapeutic agents still suffer from a number of problems, include lack of drug effectiveness, lack of selectivity, debilitating side effects, and development of drug resistance. Thus, there is a continuing need for safer and more effective treatments for cancer. Over many decades, a significant number of clinically effective anticancer agents have been successfully developed from natural products and their derivatives^{1,2}. Such plant-derived agents, i.e. the taxanes, the *bis*-indole alkaloids, and the camptothecins, are currently being widely used today as cancer chemotherapeutics, and natural products continue to be a rich source of new anticancer therapies³.

The sesquiterpene lactones are biologically active natural products that have been isolated from a variety of medicinal plants. Several compounds in this class, e.g. parthenolide (PTL, **A**, Fig 1)⁴, costunolide (**B**, Fig 1)⁵ and helenalin (**C**, Fig 1)⁶ have been studied extensively as anti-cancer agents. PTL has been identified as an antileukemic agent,⁷ and promotes apoptosis of leukemia stem cells by inhibiting the activity of the NF κ B transcription factor complex, leading to down-regulation of antiapoptotic genes under NF κ B control.⁸ The anti-cancer activity of PTL is believed to be due not only to its pro-apoptotic action, which causes the activation of p53⁹ and the enhancement of reactive oxygen species in tumor cells, but also to its ability to ablate glutathione function in hematological tumor stem cells.^{10,11}

Another example of a biologically active sesquiterpene lactone is melampomagnolide B (MMB, **D**, Fig 1), which is a constituent isolated from *Magnolia grandiflora*.¹² MMB can also be synthesized from PTL via selenium oxide oxidation of the C10 methyl group of PTL¹³ with concomitant geometrical isomerism of the C1-C10 double bond from *E* to *Z*. MMB has similar antileukemic activity as PTL, and is believed to inhibit both the NF κ B and glutathione pathways.^{14,15} Unlike PTL, the presence of the primary 14-hydroxy group in MMB enables the facile synthesis of a wide variety of conjugated C10 MMB analogs. Nasim *et al*¹⁴ have reported on a biotin conjugate of MMB, which was utilized to demonstrate that the antileukemic activity of MMB is mediated via the same mechanisms as those for PTL.¹⁵ We have previously reported on the synthesis of a series of carbamate

esters of MMB (**E**, Fig. 1), that are potent cytotoxic agents against both hematological and solid tumor cancer cells lines,¹⁶ and have also shown that dimeric carbamate and carbonate ester analogs of MMB (**F** and **G**, Fig. 1) exhibit nanomolar cytotoxicity against a wide range of human cancer cells.¹⁷ Recently, we have shown that the MMB imidazolecarbamate analog **H** (Fig. 1) has improved cytotoxicity and oral bioavailability in rats when compared to both PTL and MMB.¹⁸

The current study focuses on the synthesis of a series of novel succinamide derivatives of MMB by coupling of MMB 14-*O*-succinate¹⁹ (**2**) with various heterocyclic amines (Scheme 1 and Table 1). MMB 14-*O*-succinate was also conjugated with a variety of terminal diaminoalkanes to afford dimeric succinamide analogs of MMB (Scheme 2 and Table 2). These newly synthesized conjugates were screened for anti-cancer activity against a panel of 60 human cancer cell lines, and against M9-ENL1 acute myelogenous leukemia cells.

2. Chemistry

MMB (Scheme 1) was synthesized from PTL via selenium oxide oxidation of the C10 allylic methyl group utilizing a modification of the method of Macias *et al.*¹³ MMB was then reacted with succinic anhydride in the presence of dichloromethane/triethylamine at ambient temperature for 24 h to afford hemi-succinate ester of MMB (**2**)¹⁹ in 85% yield. Compound **2** was then reacted with a variety of heterocyclic amines in the presence of EDC, HOBt and triethylamine to afford the corresponding carboxamido derivatives **3a–3l** (Scheme 1, Table 1). Similarly, compound **2** was reacted with a variety of terminal diaminoalkanes under similar amide coupling reaction conditions to obtain the corresponding succinamide dimers **4a–4h** (Scheme 2, Table 2). In these coupling reactions, it was found that chemical reactivity of the diaminoalkane was dependent on alkane chain length, i.e. the longer chain length diaminoalkanes were more reactive than their shorter chain homologs. In some reactions, products resulting from Michael addition chemistry from the reaction of the diaminoalkane reactant at the exocyclic double bond (C-13) of MMB were observed. However, under the reaction conditions employed, these products were usually obtained in 5% yield. All reactions were carried out at ambient temperature and the desired conjugates were obtained in yields ranging from 50–89% (Tables 1 and 2).

3. Anti-cancer activity of succinamide analogs of MMB against a panel of human hematological and solid tumor cell lines

The above succinate analogs of MMB were evaluated for growth inhibition properties against a panel of 60 cell lines derived from nine human cancer types grouped into disease sub-panels that represent leukemia, lung, colon, central nervous system (CNS), melanoma, renal, ovary, breast, and prostate cancer cells. Growth inhibitory (GI⁵⁰) effects were measured as a function of the variation of optical density as a percentage of control.^{20–22}

Initial screening assays were carried out at a single concentration of 10 μ M. Four analogs, dimeric succinamido analogs **4f**, and **4g**, and succinamide analogs **3d** and **3i**, were identified as hits based on their ability to inhibit by 60% the growth of at least 8 of the 60 tumor cell lines in the panel. These four analogs were then evaluated in 5-dose assays over the

concentration range 10^{-4} – 10^{-8} μ M, and their GI_{50} values against the tumor cell lines in the panel determined (Table 3). The two dimeric analogs, **4f** and **4g**, were identified as the most potent compounds in this series of MMB analogs. The dimeric succinamido analog **4g** exhibited cytotoxicity against all the cells in the leukemia subpanel, with GI_{50} values in the nanomolar range (280–640 nM). Compound **4g** also showed nanomolar activity against solid tumor cell lines NCI-H522 (non-small cell lung cancer), HCT-116 and SW-620 (colon cancer), LOX IMVI (melanoma), and MCF7, BT-549 and MDA-MB-468 (breast cancer) cell lines with GI_{50} values of 420, 860, 540, 370, 420, 660 and 980 nM, respectively. This compound exhibited GI_{50} values <5 μ M against 90% of all the human cancer cell lines in the screening panel. Succinamido dimer **4f** was also identified as a promising anti-leukemic agent against cell lines CCRF-CEM, HL-60(TB), K-562 and SR with GI_{50} values of 320, 630, 970 and 410 nM. This compound also exhibited promising cytotoxicity against solid tumor cell lines NCI-H522 (non-small cell lung cancer), RXF 393 (renal cancer) and MCF7 (breast cancer) cells with GI_{50} values of 480, 850 and 770 nM, respectively. Compound **4f** exhibited GI_{50} values <5 μ M against 85% of all the human cancer cell lines in the screening panel.

The monomeric succinamide MMB conjugates **3d** and **3i** were less potent than the dimeric analogs showing growth inhibition activity generally in the low micromolar range ($GI_{50} = 1$ – 10 μ M) against most (75%) of the cell lines in the human cancer cell panel. Compound **3d** exhibited GI_{50} values of 1.54 and 1.60 μ M against MDA-MB-468 (breast) and NCI H522 cancer cells; whereas, compound **3i** afforded GI_{50} values of 1.06, 1.30, 1.40, and 1.50 μ M against MDA-MB-468, PC-3 (prostate), CCRF-CEM, and NCI H522 cancer cell lines.

4. Apoptotic activity of succinamide analogs of MMB against M9-ENL1 cell lines

The monomeric and dimeric MMB succinamide analogs **3a**, **3c–3l**, and **4b–4h** were also screened for apoptotic activity against the M9-ENL1 cell line obtained from transduction of human blood cells with the leukemia-derived MLL-ENL translocation gene²³. This cell line was selected for screening due to its high frequency of leukemia-initiating cells and other stem cell properties that are maintained in culture. For each compound, *in vitro* cytotoxicity studies were performed over a range of concentrations (1.25–20 μ M). Cells were plated at a density of 10^6 cells/mL in alpha-MEM culture media (Invitrogen) supplemented with 5% human plasma, 20% FBS, and the cytokines SCF, IL-3, IL-7, and FLT3 (PeproTech). Drugs were diluted from a DMSO stock into PBS such that the final concentration of DMSO did not exceed 0.5%. Evaluations were performed after 24 h of drug exposure using viability labeling with trypan blue dye, as well as flow cytometric analysis by labeling with Annexin V and propidium iodide or 7-aminoactinomycin D to delineate apoptotic cell populations and to identify the percentage of non-apoptotic cells, which was defined as the population of cells with negative staining for both labels. The percentage of non-apoptotic cells observed was normalized to that of the vehicle control, and dose-response curves were analyzed using GraphPad Prism software to determine LC_{50} values. All analyses were conducted in triplicate. In all these studies, PTL and MMB were included as reference standards. As

expected, relative cytotoxicity varied as a function of the specific modifications made to the MMB scaffold (Fig. 2).

In the monomeric series (Table 1), the 4-methylpiperidine analog **3i** exhibited about two-fold greater cytotoxicity when compared to PTL, while the structurally related analogs **3h** and **3j** were equipotent with PTL. The remaining compounds in this series exhibited less cytotoxicity when compared PTL. In the dimeric series of compounds, dimer **4g** showed similar cytotoxicity when compared to PTL; the remaining compounds in this series were less potent than PTL.

Some disparity between the data for the lead dimeric compounds that emerged from the NCI leukemia cell sub-panel and the M9-ENL1 AML cellular assay is apparent. While lead monomeric compounds **3d** and **3i** afforded similar values in the leukemia cell panel and M9-ENL1 assays (average GI₅₀ values of 6.5 and 3.1, and LC₅₀ values of 14 mM and 4.1 mM, respectively), lead dimeric compounds **4g** and **4f** exhibited average GI₅₀ values of 0.44 and 1.0 μ M in the leukemia cell panel assay and LC₅₀ values of 6.5 and 11 μ M in the M9-ENL1 assay. In this respect, growth inhibition (GI) typically requires much less drug than cell death (LC), which is what is measured in the M9-ENL1 assay, and is a function of the assay employed. In addition, another confounding factor is that all cell lines have their own unique properties, and the M9-ENL1 is quite different than most of the cell lines used in the 60-cell leukemia cell panel. We utilized the M9-ENL1 cell line because it closely parallels the behavior of primary AML cells from patients. The cell lines from the leukemia cell subpanel come mostly from a cross section of hematologic malignancies (e.g. T-ALL, CML, myeloma, etc.), but as biological surrogates; thus, they are appropriate for initial screening of large numbers of compounds.

5. Molecular Modeling Analysis

Both PTL and MMB inhibit the NF κ B transcription factor complex, resulting in down-regulation of anti-apoptotic genes under NF κ B control.^{14, 15, 24–26} From Western blot analyses, streptavidin pull-down and LC/MS/MS peptide sequencing studies with an MMB-biotin probe, we have shown that both IKK β and p65 proteins of the NF κ B pathway are identified as potential targets in the mechanism of action of MMB.^{14, 16}

Nuclear transcription factor IKK β activation plays an important role in both cancer and inflammation.^{24–26} IKK is the multiprotein complex that consists of two kinase sub-units (IKK β and IKK α) and one regulatory subunit (IKK γ) that stimulates NF κ B activation²⁷. Among the three protein subunits of the IKK complex, IKK β is mainly responsible for carrying out the signaling for the activation of NF κ B.²⁸ Inhibition of the IKK β -mediated NF κ B signaling pathway by MMB analogs would lead to decreased phosphorylation of I κ B and reduced DNA binding of p50/p65. Thus, determining the binding characteristics of the above succinamido MMB analogs with the IKK β transcription factor would help in understanding their mechanism of action at the molecular level.

IKK β (PDBID: 4KIK) inhibitors are thought to bind to the *N*-terminal kinase domain at the conserved Cys-46 residue.²⁹ This binding site is distal from the ATP-binding site, and such

inhibitors would not have to compete with the naturally occurring substrate ATP to bind. The Cys-46 residue is essential for IKK β kinase activity, and has been identified as the drug binding site for inhibition of the IKK β -mediated signaling pathway³⁰. Inhibitors that selectively bind to the conserved Cys-46 are expected to inhibit all the pathways that result in NF κ B activation.^{30, 31}

We performed covalent docking protocols with compounds **4g**, **4f**, **3h** and **3i** using the Schrodinger covalent docking suit.³² This covalent docking method first identifies the possibility of association of the active amino acid residues with the ligands. The reactive residue is then mutated to an alanine to avoid the side chain influence on the docking procedure. After that, ligands are docked at the active binding site using Glide protocol and best docking poses then identified. The side chain of the reactive residues is then restored to the original residue and best docking poses for the reaction are identified. The reaction is determined, and covalent bond is formed *in silico*. Then, the ligand and the reactive residue are minimized at the docking site. All the poses from the ligand are then clustered, and the best pose with minimum prime energy is identified for the final optimization. Affinity score is then calculated to determine the probability of the ligand and receptor interaction.

All four succinamide derivatives of MMB that demonstrated cytotoxicity against both hematological and solid tumor cell lines were used for the covalent docking analysis, and molecular interactions were further analyzed. The succinamide derivatives of MMB all covalently interacted with the active Cys-46 residue of the terminal lobe (1–109) of human IKK β via a Michael addition reaction. Compound **4g** formed a covalent interaction with Cys-46 and formed hydrogen bonding interactions with Asn-28, Pro-88, and Glu-49. The covalent bond distance of **4g** and Cys-46 was 4.207 Å and the docking score was –3.489. Compound **4f** also formed a covalent interaction with Cys-46 and formed four hydrogen bonding interaction with Asn-28, Glu-49, Ser-51 and Arg-57. The covalent bond distance of **4f** and Cys-46 was 4.746 Å and the docking score was –3.377. Compound **3i** also formed a covalent interaction with Cys-46 and formed a hydrogen bonding interaction with Glu-49. The covalent bond distance for **3i** and Cys-46 was 4.338 Å and the docking score was –2.426. Compound **3h** also formed a covalent interaction with Cys-46 but did not form any hydrogen bonding interactions; however, it formed several van-der Waals interactions. The covalent bond distance for **3h** and Cys-46 was 4.507 Å and the docking score was –1.634.

Since analogs **4g** and **4f** are dimers, these compounds form several hydrogen bonding interactions with human IKK β . Both compounds interact with Asn-28 and Glu-49. However, other interactions are different for both compounds since they interact with different parts of the binding pocket; i.e. **4g** interacts with Pro-88 and **4f** interacts with Ser-52 and Arg-57. The docking score for **4g** (–3.489) is slightly higher compared to that for **4f** (–3.377); and the covalent bond length of Cys-46 and **4g** interaction (4.204 Å) is stronger than the Cys-46 and **4f** interaction (4.746 Å).

The non-dimeric analogs **3i** and **3h** form fewer hydrogen bonding interactions with human IKK β . Both compounds form stable covalent interactions with Cys-46. However, **3i** makes a stronger bond with Cys-46 (4.338 Å bond length) when compared to that for **3h** (4.507 Å bond length). The docking score for **3i** (–2.426) is comparatively better than that for **3h**

(−1.643). In addition, **3i** forms a hydrogen bonding interaction Glu-49. All four analogs afford stable interactions with human IKK β , and all analogs exhibit similar potential energy patterns during their bound state with human IKK β .

6. Summary

In summary, we have synthesized and identified a small library of succinamide derivatives of MMB that exhibit cytotoxicity against both hematological and solid tumor cell lines. Four of these analogs: **3d**, **3i**, **4f** and **4g** were identified as molecules of interest. The most potent compounds, **4f** and **4g** exhibited anticancer activity in the nanomolar range (GI_{50} = 280–980 nM) against several human cancer cell lines in the panel. By comparison, compounds **3d** and **3i** were less active than the dimeric analogs, and showed anti-cancer activity in the low micromolar range (GI_{50} = 1.06–5.00 μ M) against most of the cancer cell lines in the panel.

The above compounds were also evaluated for apoptotic activity against the M9-ENL1 acute myelogenous leukemia cell line; compounds **3h–3j** and **4g** were similar in potency to parthenolide (LC_{50} = 7.5 μ M), exhibiting LC_{50} values in the range 4.1–8.1 μ M. Molecular docking studies indicate that these molecules interact covalently with the highly conserved Cys-46 residue of the *N*-terminal lobe (1–109) of human IKK β causing inhibition of the NF κ B transcription factor complex, which results in down-regulation of anti-apoptotic genes under NF κ B control.

7. Experimental

All synthesized compounds were purified by column chromatography (silica gel; methanol/dichloromethane) to afford pure products (>98% purity) in 50–89 % yield. Melting points were recorded on a Kofler hot-stage apparatus and are uncorrected. The synthesized compounds were fully characterized by 1H NMR and ^{13}C NMR spectral analysis which were recorded on a Varian 400 MHz spectrometer equipped with a Linux workstation running on vNMRj software. HRMS data were obtained on an Agilent 6210 LCTOF instrument operating in multimode.

The M9-ENL1 cell line was obtained from transduction of human blood cells with the leukemia-derived MLL-ENL translocation gene.²³ The methodology utilized in the anti-cancer screening assay utilized the reported literature procedure, which can be found at <http://dtp.nci.nih.gov/branches/btb/ivclsp.html>.

7.1. General synthetic procedure and analytical data for succinamide derivatives of MMB

To a stirred solution of MMB 14-*O*-succinate (**2**, 50 mg, 0.14 mmol) in dichloromethane was added EDC (40.26 mg, 0.21 mmol), HOBt (28.35 mg, 0.21 mmol), triethylamine (42.42 mg, 0.42 mmol) and the appropriate heterocyclic amine (0.14 mmol) at ambient temperature. The reaction mixture was stirred for 3–12 h at ambient temperature. When the reaction was complete (monitored by TLC), water was added and the resulting aqueous mixture was extracted with dichloromethane (2x10 mL). The organic layers were combined, washed with water followed by brine solution, and dried over anhydrous Na_2SO_4 . The solvent was then evaporated under reduced pressure to afford the crude product. The crude

product was purified by column chromatography (silica gel, 3–5% methanol in dichloromethane) to afford the desired succinamide analog as a white solid.

7.1.1. ((1aR,7aS,10aS,10bS,E)-1a-methyl-8-methylene-9-oxo-1a,2,3,6,7,7a,8,9,10a,10b-decahydrooxireno[2',3':9,10]cyclodeca[1,2-b]furan-5-yl)methyl-4-((2-morpholinoethyl)amino)-4-oxobutanoate (3a)—¹H NMR (CDCl₃, 400 MHz): δ 6.24 (d, *J* = 3.6 Hz, 1H), 6.11 (brs, 1H), 5.68 (t, *J* = 7.6 Hz, 1H), 5.59 (d, *J* = 2.8 Hz, 1H), 4.66 (d, *J* = 12.8 Hz, 1H), 4.50 (d, *J* = 12.4 Hz, 1H), 3.84 (t, *J* = 9.2 Hz, 1H), 3.72 (t, *J* = 4 Hz, 4H), 3.37-3.33 (q, *J* = 5.6, 10.8 Hz, 2H), 2.97-2.92 (m, 1H), 2.87 (d, *J* = 9.6 Hz, 1H), 2.65 (t, *J* = 6 Hz, 2H), 2.52-2.14 (m, 14 H), 1.72-1.60 (m, 1H), 1.54 (s, 3H), 1.11 (t, *J* = 11.6 Hz, 1H) *ppm*. ¹³C NMR (CDCl₃, 100 MHz): δ 172.6, 170.8, 169.3, 138.6, 134.6, 130.2, 120.1, 80.9, 66.8, 66.6, 63.0, 59.7, 56.8, 53.1, 42.4, 36.4, 35.4, 30.4, 29.0, 25.5, 24.3, 23.6, 17.8 *ppm*. HRMS (ESI) *m/z* calcd for C₂₅H₃₇N₂O₇ (M + H)⁺ 477.2595, found 477.2589.

7.1.2. ((1aR,7aS,10aS,10bS,E)-1a-methyl-8-methylene-9-oxo-1a,2,3,6,7,7a,8,9,10a,10b-decahydrooxireno[2',3':9,10]cyclodeca[1,2-b]furan-5-yl)methyl-4-((3-(1H-imidazol-1-yl)propyl)amino)-4-oxobutanoate (3b)—¹H NMR (CDCl₃, 400 MHz) δ 7.52 (s, 1H), 7.05 (s, 1H), 6.94 (s, 1H), 6.23 (d, *J* = 2.8 Hz, 1H), 5.77 (brs, 1H), 5.67 (t, *J* = 8.0 Hz, 1H), 5.56 (d, *J* = 3.2 Hz, 1H), 4.64 (d, *J* = 12.4 Hz, 1H), 4.51 (d, *J* = 12.4 Hz, 1H), 3.99 (t, *J* = 6.8 Hz, 2H), 3.84 (t, *J* = 9.2 Hz, 1H), 3.26 (q, *J* = 12.8 Hz, 2H), 2.99-2.93 (m, 1H), 2.86 (d, *J* = 9.2 Hz, 1H), 2.65 (t, *J* = 5.6 Hz, 2H), 2.49-2.12 (m, 8 H), 2.02-1.95 (m, 2H), 1.69-1.62 (m, 1H), 1.53 (s, 3H), 1.09 (t, *J* = 12 Hz, 1H) *ppm*. ¹³C NMR (CDCl₃, 100 MHz) δ 172.7, 171.4, 169.4, 138.7, 137.0, 134.5, 130.3, 129.3, 120.1, 118.7, 81.0, 66.9, 63.1, 59.8, 44.4, 42.5, 36.7, 36.5, 31.1, 30.5, 29.1, 25.6, 24.4, 23.7, 17.9 *ppm*. HRMS (ESI) *m/z* calcd for C₂₅H₃₄N₃O₆ (M + H)⁺ 472.2442, found 472.2471.

7.1.3. ((1aR,7aS,10aS,10bS,E)-1a-methyl-8-methylene-9-oxo-1a,2,3,6,7,7a,8,9,10a,10b-decahydrooxireno[2',3':9,10]cyclodeca[1,2-b]furan-5-yl)methyl-4-oxo-4-((2-(pyridin-2-yl)ethyl)amino)butanoate (3c)—¹H NMR (CDCl₃, 400 MHz) δ 8.52 (d, *J* = 4.0 Hz, 1H), 7.62 (t, *J* = 8.0 Hz, 1H), 7.16 (t, *J* = 7.6 Hz, 2H), 6.59 (s, 1H), 6.23 (d, *J* = 3.2 Hz, 1H), 5.66 (t, *J* = 8.0 Hz, 1H), 5.59 (d, *J* = 2.8 Hz, 1H), 4.67 (d, *J* = 12.8 Hz, 1H), 4.48 (d, *J* = 12.8 Hz, 1H), 3.84 (t, *J* = 9.2 Hz, 1H), 3.65 (q, *J* = 12 Hz, 2H), 2.98-2.83 (m, 4 H), 2.63 (t, *J* = 6.4 Hz, 2H), 2.48-2.12 (m, 7H), 1.85 (brs, 1H), 1.64 (t, *J* = 12.8 Hz, 1H), 1.53 (s, 3H), 1.10 (t, *J* = 11.6 Hz, 1H) *ppm*. ¹³C NMR (CDCl₃, 100 MHz) δ 172.8, 171.0, 169.5, 159.6, 149.2, 138.8, 136.9, 135.0, 130.5, 123.6, 121.7, 120.5, 81.1, 67.1, 63.4, 60.0, 42.7, 38.8, 36.8, 36.7, 30.8, 29.4, 25.8, 24.5, 23.9, 18.1 *ppm*. HRMS (ESI) *m/z* calcd for C₂₆H₃₃N₂O₆ (M + H)⁺ 469.2333, found 469.2421.

7.1.4. ((1aR,7aS,10aS,10bS,E)-1a-methyl-8-methylene-9-oxo-1a,2,3,6,7,7a,8,9,10a,10b-decahydrooxireno[2',3':9,10]cyclodeca[1,2-b]furan-5-yl)methyl-4-(((6-chloropyridin-3-yl)methyl) amino)-4-oxobutanoate (3d)—¹H NMR (CDCl₃, 400 MHz) δ 8.28 (s, 1H), 7.61 (d, *J* = 8.4 Hz, 1H), 7.29 (d, *J* = 8.4 Hz, 1H), 6.22 (d, *J* = 3.2 Hz, 1H), 6.07 (s, 1H), 5.68 (t, *J* = 7.2 Hz, 1H), 5.55 (s, 1H), 4.65 (d, *J* = 12.4 Hz, 1H), 4.49 (d, *J* = 12.8 Hz, 1H), 4.42 (d, *J* = 5.6 Hz, 2H), 3.85 (t, *J* = 9.6 Hz, 1H), 2.95 (t, *J* = 8.8 Hz, 1H), 2.85 (d, *J* = 9.6 Hz, 1H), 2.68 (t, *J* = 6 Hz, 2H), 2.52-2.11 (m, 7H), 1.67-1.62 (m, 2H),

1.53 (s, 3H), 1.12 (t, $J = 11.6$ Hz, 1H) *ppm*. ^{13}C NMR (CDCl_3 , 100 MHz) δ 172.8, 171.5, 169.6, 150.7, 148.9, 138.9, 138.6, 134.8, 133.1, 130.8, 124.4, 120.4, 81.2, 67.2, 63.3, 60.1, 42.7, 40.5, 36.7, 30.7, 29.3, 25.8, 24.6, 23.9, 18.1 *ppm*. HRMS (ESI) m/z calcd for $\text{C}_{25}\text{H}_{30}\text{ClN}_2\text{O}_6$ ($\text{M} + \text{H}$) $^+$ 489.1787, found 489.1792.

7.1.5. ((1a*R*,7a*S*,10a*S*,10b*S*,*E*)-1a-methyl-8-methylene-9-oxo-1a,2,3,6,7,7a,8,9,10a,10b-decahydrooxireno[2',3':9,10]cyclodeca[1,2-*b*]furan-5-yl)methyl-4-oxo-4-((pyridin-2-ylmethyl) amino)butanoate (3e)— ^1H NMR (CDCl_3 , 400 MHz): δ 8.55 (d, $J = 4.8$ Hz, 1H), 7.73 (t, $J = 7.2$ Hz, 1H), 7.32 (7.32 (d, $J = 8$ Hz, 1H), 7.26 (d, $J = 8.4$ Hz 1H), 6.92 (brs, 1H), 6.24 (d, $J = 3.2$ Hz, 1H), 5.69 (t, $J = 7.6$ Hz, 1H), 5.59 (d, $J = 3.2$ Hz, 1H), 4.70 (d, $J = 12.4$ Hz, 1H), 4.58 (d, $J = 5.2$ Hz, 2H), 4.49 (d, $J = 12.8$ Hz, 1H), 3.85 (t, $J = 8.8$ Hz, 1H), 2.95-2.85 (m, 2H), 2.68-2.62 (m, 4 H), 2.45-2.13 (m, 6H), 1.65 (t, $J = 13.2$ Hz, 1H), 1.55 (s, 3H), 1.11 (t, $J = 11.6$ Hz, 1H) *ppm*. ^{13}C NMR (CDCl_3 , 100 MHz): δ 172.7, 171.2, 169.6, 155.9, 148.4, 138.8, 137.7, 134.9, 130.7, 122.8, 122.6, 120.5, 81.1, 67.1, 63.4, 60.1, 44.2, 42.7, 36.7, 30.7, 29.4, 25.9, 24.6, 23.9, 18.1 *ppm*. HRMS (ESI) m/z calcd for $\text{C}_{25}\text{H}_{31}\text{ClN}_2\text{O}_6$ ($\text{M} + \text{H}$) $^+$ 455.2177, found 455.2212.

7.1.6. ((1a*R*,7a*S*,10a*S*,10b*S*,*E*)-1a-methyl-8-methylene-9-oxo-1a,2,3,6,7,7a,8,9,10a,10b-decahydrooxireno[2',3':9,10]cyclodeca[1,2-*b*]furan-5-yl)methyl-4-((5-(methylthio)-1*H*-1,2,4-triazol-3-yl)amino)-4-oxobutanoate (3f)— ^1H NMR (CDCl_3 , 400 MHz): δ 6.39 (s, 2H), 6.22 (s, 1H), 5.72 (t, $J = 7.6$ Hz, 1H), 5.54 (s, 1H), 4.74 (d, $J = 12.4$ Hz, 1H), 4.48 (d, $J = 12.4$ Hz, 1H), 3.84 (t, $J = 9.2$ Hz, 1H), 3.31-3.26 (m, 2H), 2.95 (t, $J = 4.8$ Hz, 1H), 2.86 (d, $J = 9.2$ Hz, 1H), 2.70 (t, $J = 6.4$ Hz, 2H), 2.52 (s, 3H), 2.46-2.12 (m, 6H), 1.66 (t, $J = 11.2$ Hz, 1H), 1.53 (s, 3H), 1.10 (t, $J = 12.4$ Hz, 1H) *ppm*. ^{13}C NMR (CDCl_3 , 100 MHz): δ 172.3, 171.9, 169.6, 162.4, 156.7, 139.0, 134.7, 131.6, 120.2, 81.1, 67.2, 63.3, 60.0, 42.7, 36.6, 30.3, 27.9, 25.9, 24.9, 24.0, 18.1, 13.8 *ppm*. HRMS (ESI) m/z calcd for $\text{C}_{22}\text{H}_{29}\text{N}_4\text{O}_6$ ($\text{M} + \text{H}$) $^+$ 477.1802, found 477.1836.

7.1.7. ((1a*R*,7a*S*,10a*S*,10b*S*,*E*)-1a-methyl-8-methylene-9-oxo-1a,2,3,6,7,7a,8,9,10a,10b-decahydrooxireno[2',3':9,10]cyclodeca[1,2-*b*]furan-5-yl)methyl(3-morpholinopropyl)carbamate (3g)— ^1H NMR (CDCl_3 , 400 MHz): δ 7.05 (brs, 1H), 6.24 (s, 1H), 5.67 (t, $J = 6.8$ Hz, 1H), 5.59 (s, 1H), 4.64 (d, $J = 12.4$ Hz, 1H), 4.51 (d, $J = 12.4$ Hz, 1H), 3.84 (t, $J = 9.2$ Hz, 1H), 3.75 (brs, 4H), 3.35 (d, $J = 4.8$ Hz, 2H), 2.96 (t, $J = 3.0$ Hz, 1H), 2.86 (d, $J = 9.2$ Hz, 1H), 2.65 (t, $J = 6.8$ Hz, 2H), 2.52-2.13 (m, 14H), 1.69-1.63 (m, 3H), 1.54 (s, 3H), 1.11 (t, $J = 12.4$ Hz, 1H) *ppm*. ^{13}C NMR (CDCl_3 , 100 MHz): δ 172.9, 171.0, 169.6, 138.9, 134.8, 130.2, 120.4, 81.2, 67.0, 66.8, 63.3, 60.1, 57.6, 53.6, 42.7, 39.2, 36.7, 30.8, 29.3, 25.8, 24.6, 24.6, 23.9, 18.1 *ppm*. HRMS (ESI) m/z calcd for $\text{C}_{26}\text{H}_{39}\text{N}_2\text{O}_7$ ($\text{M} + \text{H}$) $^+$ 491.2752, found 491.2772.

7.1.8. ((1a*R*,7a*S*,10a*S*,10b*S*,*E*)-1a-methyl-8-methylene-9-oxo-1a,2,3,6,7,7a,8,9,10a,10b-decahydrooxireno[2',3':9,10]cyclodeca[1,2-*b*]furan-5-yl)methyl-4-(4,4-difluoropiperidin-1-yl)-4-oxobutanoate (3h)— ^1H NMR (CDCl_3 , 400 MHz) δ 6.24 (d, $J = 3.6$ Hz, 1H), 5.70 (t, $J = 7.6$ Hz, 1H), 5.59 (d, $J = 2.4$ Hz, 1H), 4.70 (d, $J = 12.4$ Hz, 1H), 4.50 (d, $J = 12$ Hz, 1H), 3.85 (t, $J = 9.2$ Hz, 1H), 3.71 (t, $J = 5.2$ Hz, 2H), 3.59 (t, $J = 5.6$ Hz, 2H), 2.97 (t, $J = 9.6$ Hz, 1H), 2.88 (d, $J = 9.6$ Hz, 1H), 2.65 (s, 4H), 2.45-2.13 (m,

6H), 2.05-1.91 (m, 4 H), 1.69-1.63 (m, 1H), 1.54 (s, 3H), 1.11 (t, $J = 12$ Hz, 1H) *ppm*. ^{13}C NMR (CDCl_3 , 100 MHz) δ 172.9, 169.6, 138.9, 135.0, 130.7, 120.4, 81.2, 67.2, 63.4, 60.0, 42.7, 41.3, 39.4, 36.7, 34.1, 29.2, 27.8, 25.9, 24.7, 23.9, 18.1 *ppm*. HRMS (ESI) m/z calcd for $\text{C}_{24}\text{H}_{32}\text{F}_2\text{NO}_6$ ($\text{M} + \text{H}$) $^+$ 468.2192, found 468.2184.

7.1.9. ((1a*R*,7a*S*,10a*S*,10b*S*,*E*)-1a-methyl-8-methylene-9-oxo-1a,2,3,6,7,7a,8,9,10a,10b-decahydrooxireno[2',3':9,10]cyclodeca [1,2-*b*]furan-5-yl)methyl-4-(4-methylpiperidin-1-yl)-4-oxobutanoate (3i)— ^1H NMR (CDCl_3 , 400 MHz): δ 6.24 (d, $J = 2.8$ Hz, 1H), 5.69 (t, $J = 8.4$ Hz, 1H), 5.61 (s, 1H), 4.70 (d, $J = 12$ Hz, 1H), 4.502 (t, $J = 14.4$ Hz, 2H), 3.87-3.80(m, 2H), 3.03-2.92 (m, 2H), 2.88 (d, $J = 9.6$ Hz, 1H), 2.62-2.13 (m, 11H), 1.71-1.63 (m, 4H), 1.54(s, 3H), 1.14-1.04 (m, 3H), 0.95 (d, $J = 6.4$ Hz, 3H) *ppm*. ^{13}C NMR (CDCl_3 , 100 MHz): δ 173.2, 169.6, 169.2, 138.9, 135.1, 130.4, 120.6, 81.3, 67.1, 63.4, 60.0, 45.7, 42.8, 42.3, 36.7, 34.6, 33.8, 31.2, 29.4, 28.1, 25.9, 24.6, 23.9, 21.8, 18.1 *ppm*. HRMS (ESI) m/z calcd for $\text{C}_{25}\text{H}_{36}\text{NO}_6$ ($\text{M} + \text{H}$) $^+$ 446.2537, found 446.2540.

7.1.10. ((1a*R*,7a*S*,10a*S*,10b*S*,*E*)-1a-methyl-8-methylene-9-oxo-1a,2,3,6,7,7a,8,9,10a,10b-decahydrooxireno[2',3':9,10]cyclodeca[1,2-*b*]furan-5-yl)methyl-4-(4-methylpiperazin-1-yl)-4-oxobutanoate (3j)— ^1H NMR (CDCl_3 , 400 MHz): δ 6.24 (d, $J = 3.2$ Hz, 1H), 5.69 (t, $J = 8.4$ Hz, 1H), 5.61 (d, $J = 3.2$ Hz, 1H), 4.70 (d, $J = 12.4$ Hz, 1H), 4.50 (d, $J = 12.4$ Hz, 1H) 3.85 (t, $J = 9.2$ Hz, 1H), 3.61(s, 2H), 3.49 (t, $J = 4.8$ Hz, 2H), 2.95 (t, $J = 8.4$ Hz, 1H), 2.88 (d, $J = 9.6$ Hz, 1H), 2.63 (s, 4H), 2.45-2.13 (m, 13H), 1.68 (t, $J = 11.6$ Hz, 1H), 1.54 (s, 3H), 1.11 (t, $J = 11.6$ Hz, 1H) *ppm*. ^{13}C NMR (CDCl_3 , 100 MHz): δ 173.1, 169.6, 169.4, 138.9, 135.1, 130.4, 120.5, 81.2, 67.1, 63.4, 60.0, 55.1, 54.7, 46.2, 45.2, 42.8, 41.8, 36.7, 29.2, 28.0, 25.9, 24.6, 23.9, 18.1 *ppm*. HRMS (ESI) m/z calcd for $\text{C}_{24}\text{H}_{35}\text{N}_2\text{O}_6$ ($\text{M} + \text{H}$) $^+$ 447.2490, found 447.2497.

7.1.11. ((1a*R*,7a*S*,10a*S*,10b*S*,*E*)-1a-methyl-8-methylene-9-oxo-1a,2,3,6,7,7a,8,9,10a,10b-decahydrooxireno[2',3':9,10]cyclodeca[1,2-*b*]furan-5-yl)methyl-4-((3-amino-1*H*-1,2,4-triazol-5-yl)amino)-4-oxobutanoate (3k)— ^1H NMR (CDCl_3 , 400 MHz): δ 6.67 (s, 2H), 6.09 (d, $J = 3.2$ Hz, 1H), 5.58 (t, $J = 8.0$ Hz, 1H), 5.45 (d, $J = 2.8$ Hz, 1H), 4.59 (d, $J = 12.4$ Hz, 1H), 4.36-4.32 (m, 3H), 3.74 (t, $J = 9.2$ Hz, 1H), 3.02 (t, $J = 5.6$ Hz, 2H), 2.83 (s, 1H), 2.74 (d, $J = 9.6$ Hz, 1H), 2.55 (t, $J = 6.4$ Hz, 2H), 2.32-2.00 (m, 6H), 1.54 (t, $J = 10.8$ Hz, 1H), 1.41 (s, 3H), 0.97 (t, $J = 12.8$ Hz, 1H) *ppm*. ^{13}C NMR (CDCl_3 , 100 MHz) δ 172.1, 171.9, 169.8, 157.1, 138.9, 134.7, 131.5, 120.3, 81.1, 67.5, 63.3, 60.2, 42.7, 36.5, 30.1, 28.0, 25.8, 24.9, 23.9, 18.0 *ppm*. HRMS (ESI) m/z calcd for $\text{C}_{21}\text{H}_{28}\text{N}_5\text{O}_6$ ($\text{M} + \text{H}$) $^+$ 446.2034 found 446.2078.

7.1.12. ((1a*R*,7a*S*,10a*S*,10b*S*,*E*)-1a-methyl-8-methylene-9-oxo-1a,2,3,6,7,7a,8,9,10a,10b-decahydrooxireno[2',3':9,10]cyclodeca [1,2-*b*]furan-5-yl)methyl-4-((6-aminopyridin-2-yl)amino)-4-oxobutanoate (3l)— ^1H NMR (CDCl_3 , 400 MHz): δ 7.70 (s, 1H), 7.45-7.41 (m, 2H), 6.25-6.22 (m, 2H), 5.67 (t, $J = 8.8$ Hz, 1H), 5.56 (d, $J = 3.2$ Hz, 1H), 4.72 (d, $J = 12.8$ Hz, 1H), 4.49 (d, $J = 12.8$ Hz, 1H), 4.30 (s, 2H), 3.83 (t, $J = 9.6$ Hz, 1H), 2.91-2.83 (m, 2H), 2.74-2.68 (m, 4H), 2.43-2.11(m, 6H), 1.65-1.56 (m, 1H), 1.53 (s, 3H), 1.09 (t, $J = 11.6$ Hz, 1H) *ppm*. ^{13}C NMR (CDCl_3 , 100 MHz): δ 172.5, 169.5, 157.2, 149.6, 140.2, 138.8, 134.9, 130.6, 120.6, 120.5 104.4, 103.2, 81.2, 67.2, 63.4, 60.0, 42.7,

36.7, 32.0, 29.2, 25.8, 24.5, 23.9, 18.1 *ppm*. HRMS (ESI) *m/z* calcd for C₂₄H₃₀N₃O₆ (M + H)⁺ 456.2129, found 456.2177.

7.2. General synthetic procedure and analytical data for succinamide dimers of MMB

To a stirred solution of MMB 14-O-succinate¹⁹ (**2**, 50 mg, 0.14 mmol) in dichloromethane was added EDC (40.26 mg, 0.21 mmol), HOBt (28.35 mg, 0.21 mmol), triethylamine (42.42 mg, 0.42 mmol) and the appropriate diaminoalkane (0.07 mmol) at ambient temperature. The reaction mixture was stirred for 5–12 h at ambient temperature. When the reaction was complete (monitored by TLC), water was added and the resulting aqueous mixture was extracted with dichloromethane (2x10 mL). The organic layers were combined, washed with water followed by brine solution, and dried over anhydrous Na₂SO₄. The solvent was then evaporated under reduced pressure to afford the crude product, which was purified by column chromatography (silica gel, 1–3% methanol in dichloromethane) to afford the desired succinamide analog as a white solid.

7.2.1. bis-(((1a*R*,7a*S*,10a*S*,10b*S*,*E*)-1a-methyl-8-methylene-9-oxo-1a,2,3,6,7,7a,8,9,10a,10b-decahydrooxireno[2',3':9,10]cyclodeca[1,2-*b*]furan-5-yl)methyl)-4,4'-(propane-1,3-diyl bis-(azanediyl)) bis-(4-oxobutanoate (4a**))**—¹H NMR (CDCl₃, 400 MHz): δ 6.28 (t, *J* = 6.0 Hz, 2H), 6.24 (d, *J* = 3.6 Hz, 2H), 5.68 (t, *J* = 8.4 Hz, 2H), 5.58 (d, *J* = 3.2 Hz, 2H), 4.65 (d, *J* = 12.4 Hz, 2H), 4.51 (d, *J* = 12.8 Hz, 2H), 3.85 (t, *J* = 9.2 Hz, 2H), 3.34–3.21 (m, 4H), 3.03–2.97 (m, 2H), 2.88 (d, *J* = 9.6 Hz, 2H), 2.68–2.64 (m, 4H), 2.50–2.13 (m, 16H), 1.69–1.60 (m, 4H), 1.54 (s, 6H), 1.12 (t, *J* = 11.6 Hz, 2H) *ppm*. ¹³C NMR (CDCl₃, 100 MHz) δ 172.9, 171.8, 169.7, 138.9, 134.8, 130.2, 120.3, 81.2, 67.0, 63.2, 60.1, 42.6, 36.7, 36.4, 30.6, 29.6, 29.4, 25.8, 24.6, 23.8, 18.1 *ppm*. HRMS (ESI) *m/z* calcd for C₄₁H₅₅N₂O₁₂ (M + H)⁺ 767.3750, found 767.3767.

7.2.2. bis-(((1a*R*,7a*S*,10a*S*,10b*S*,*E*)-1a-Methyl-8-methylene-9-oxo-1a,2,3,6,7,7a,8,9,10a,10b-decahydrooxireno[2',3':9,10]cyclodeca[1,2-*b*]furan-5-yl)methyl)-4,4'-(butane-1,4-diyl bis-(azanediyl)) bis-(4-oxobutanoate) (4b**)**—¹H NMR (CDCl₃, 400 MHz): δ 6.23 (s, 2H), 6.05 (s, 2H), 5.65 (t, *J* = 8 Hz, 2H), 5.58 (s, 2H), 4.62 (d, *J* = 12.8 Hz, 2H), 4.54 (d, *J* = 13.2 Hz, 2H), 3.85 (t, *J* = 9.2 Hz, 2H), 3.30–3.09 (m, 6H), 2.90 (d, *J* = 9.6 Hz, 2H), 2.70–2.62 (m, 4H), 2.50–2.17 (m, 16H), 1.69 (t, *J* = 11.6 Hz, 2H), 1.54 (s, 6H), 1.50 (s, 4H), 1.13 (t, *J* = 12.4 Hz, 2H) *ppm*. ¹³C NMR (CDCl₃, 100 MHz): δ 173.0, 171.4, 169.8, 139.0, 134.7, 129.8, 120.3, 81.3, 66.8, 63.2, 60.2, 42.5, 39.2, 36.7, 30.6, 29.4, 26.8, 25.8, 24.7, 23.8, 18.1 *ppm*. HRMS (ESI) *m/z* calcd for C₄₂H₅₇N₂O₁₂ (M + H)⁺ 781.3906, found 781.3941.

7.2.3. bis-(((1a*R*,7a*S*,10a*S*,10b*S*,*E*)-1a-Methyl-8-methylene-9-oxo-1a,2,3,6,7,7a,8,9,10a,10b-decahydrooxireno[2',3':9,10]cyclodeca[1,2-*b*]furan-5-yl)methyl)-4,4'-(pentane-1,5-diyl bis-(azanediyl)) bis-(4-oxobutanoate) (4c**)**—¹H NMR (CDCl₃, 400 MHz): δ 6.23 (s, 2H), 5.85 (brs, 2H), 5.67 (t, *J* = 7.2 Hz, 2H), 5.59 (s, 2H), 4.64 (d, *J* = 12 Hz, 2H), 4.52 (d, *J* = 11.6 Hz, 2H), 3.85 (t, *J* = 9.6 Hz, 2H), 3.23 (brs, 4H), 3.01 (t, *J* = 9.2 Hz, 2H), 2.89 (d, *J* = 9.6 Hz, 2H), 2.66–2.63 (m, 4H), 2.48–2.14 (m, 16H), 1.66 (t, *J* = 11.6 Hz, 2H), 1.55–1.48 (m, 10 H), 1.32 (d, *J* = 6 Hz, 2H), 1.11 (t, *J* = 12 Hz, 2H) *ppm*. ¹³C NMR (CDCl₃, 100 MHz): δ 173.0, 171.3, 169.7, 139.0, 134.8, 130.1,

120.4, 81.3, 67.0, 63.3, 60.1, 42.7, 39.2, 36.7, 30.7, 29.4, 29.0, 25.9, 24.7, 23.9, 23.7, 18.1 *ppm*. HRMS (ESI) *m/z* calcd for C₄₃H₅₉N₂O₁₂ (M + H)⁺ 795.4063, found 795.4088.

7.2.4. bis-(((1*aR*,7*aS*,10*aS*,10*bS*,*E*)-1*a*-Methyl-8-methylene-9-oxo-1*a*,2,3,6,7,7*a*,8,9,10*a*,10*b*-decahydrooxireno[2',3':9,10]cyclodeca[1,2-*b*]furan-5-yl)methyl)-4,4'-(hexane-1,6-diyl bis-(azanediyl)) bis-(4-oxobutanoate) (4d)—¹H NMR (CDCl₃, 400 MHz): δ 6.24 (d, *J* = 3.2 Hz, 2H), 5.83 (brs, 2H), 5.66 (t, *J* = 7.6 Hz, 2H), 5.60 (d, *J* = 2.8 Hz, 2H), 4.64 (d, *J* = 12.8 Hz, 2H), 4.52 (d, *J* = 12.8 Hz, 2H), 3.85 (t, *J* = 9.2 Hz, 2H), 3.24-3.21 (m, 4H), 2.98 (t, *J* = 9.2 Hz, 2H), 2.87 (d, *J* = 9.2 Hz, 2H), 2.67-2.63 (m, 4H), 2.49-2.13 (m, 16H), 1.66 (t, *J* = 10.8 Hz, 2H), 1.54 (s, 6H), 1.48 (t, *J* = 6.4 Hz, 4H), 1.32 (brs, 4H), 1.11 (t, *J* = 12 Hz, 2H) *ppm*. ¹³C NMR (CDCl₃, 100 MHz): δ 172.9, 171.2, 169.7, 138.9, 134.8, 130.0, 120.4, 81.3, 66.9, 63.3, 60.1, 42.6, 39.2, 36.7, 30.7, 29.4, 29.4, 26.0, 25.8, 24.5, 23.8, 18.1 *ppm*. HRMS (ESI) *m/z* calcd for C₄₄H₆₁N₂O₁₂ (M + H)⁺ 809.4219, found 809.4215.

7.2.5. bis-(((1*aR*,7*aS*,10*aS*,10*bS*,*E*)-1*a*-Methyl-8-methylene-9-oxo-1*a*,2,3,6,7,7*a*,8,9,10*a*,10*b*-decahydrooxireno[2',3':9,10] cyclodeca[1,2-*b*]furan-5-yl)methyl)-4,4'-(heptane-1,7-diyl bis-(azanediyl)) bis-(4-oxobutanoate) (4e)—¹H NMR (CDCl₃, 400 MHz): δ 6.24 (d, *J* = 3.2 Hz, 2H), 5.67-5.65 (m, 4H), 5.60 (d, *J* = 2.8 Hz, 2H), 4.66 (d, *J* = 12.4 Hz, 2H), 4.51 (d, *J* = 12.8 Hz, 2H), 3.85 (t, *J* = 9.2 Hz, 2H), 3.24-3.19 (m, 4H), 2.95 (t, *J* = 9.2 Hz, 2H), 2.87 (d, *J* = 9.2 Hz, 2H), 2.65 (t, *J* = 6.4 Hz, 4H), 2.48-2.13 (m, 16H), 1.66 (t, *J* = 11.2 Hz, 2H), 1.54 (s, 6H), 1.48 (t, *J* = 6.8 Hz, 4H), 1.31(s, 6H), 1.11 (t, *J* = 12 Hz, 2H) *ppm*. ¹³C NMR (CDCl₃, 100 MHz): δ 172.9, 171.1, 169.6, 139.0, 134.9, 130.2, 120.4, 81.3, 67.1, 63.4, 60.0, 42.7, 39.6, 36.8, 30.8, 29.5, 28.8, 26.7, 26.2, 25.9, 24.6, 23.9, 18.1 *ppm*. HRMS (ESI) *m/z* calcd for C₄₅H₆₃N₂O₁₂ (M + H)⁺ 823.4376, found 823.4371.

7.2.6. bis-(((1*aR*,7*aS*,10*aS*,10*bS*,*E*)-1*a*-Methyl-8-methylene-9-oxo-1*a*,2,3,6,7,7*a*,8,9,10*a*,10*b*-decahydrooxireno[2',3':9,10] cyclodeca[1,2-*b*]furan-5-yl)methyl)-4,4'-(octane-1,8-diyl bis-(azanediyl)) bis-(4-oxobutanoate) (4f)—¹H NMR (CDCl₃, 400 MHz): δ 6.25 (d, *J* = 3.2 Hz, 2H), 5.69-5.67 (m, 4H), 5.61(d, *J* = 3.2 Hz, 2H), 4.67 (d, *J* = 12.4 Hz, 2H), 4.50 (d, *J* = 12.8 Hz, 2H), 3.85 (t, *J* = 8.8 Hz, 2H), 3.23-3.20 (m, 4H), 2.93 (t, *J* = 9.2 Hz, 2H), 2.87 (d, *J* = 9.2 Hz, 2H), 2.67-2.64 (m, 4H), 2.48-2.13 (m, 16H), 1.66 (t, *J* = 10 Hz, 2H), 1.54 (s, 6H), 1.47 (t, *J* = 6.8 Hz, 4H), 1.29 (s, 8H), 1.11(t, *J* = 11.6 Hz, 2H) *ppm*. ¹³C NMR (CDCl₃, 100 MHz): δ 172.9, 171.1, 169.6, 138.9, 134.9, 130.4, 120.5, 81.2, 67.1, 63.3, 60.1, 42.7, 39.7, 36.7, 30.8, 29.6, 29.4, 29.1, 26.8, 25.8, 24.5, 23.9, 18.1 *ppm*. HRMS (ESI) *m/z* calcd for C₄₆H₆₅N₂O₁₂ (M + H)⁺ 837.4532, found 837.4535.

7.2.7. bis-(((1*aR*,7*aS*,10*aS*,10*bS*,*E*)-1*a*-Methyl-8-methylene-9-oxo-1*a*,2,3, 6,7,7*a*,8,9,10*a*,10*b*-decahydrooxireno[2',3':9,10] cyclodeca[1,2-*b*]furan-5-yl)methyl)-4,4'-(nonane-1,9-diyl bis-(azanediyl)) bis-(4-oxobutanoate) (4g)—¹H NMR (CDCl₃, 400 MHz): δ 6.25 (d, *J* = 3.2 Hz, 2H), 5.69-5.65 (m, 4H), 5.61(d, *J* = 3.2 Hz, 2H), 4.68 (d, *J* = 12.8 Hz, 2H), 4.50 (d, *J* = 12.8 Hz, 2H), 3.85 (t, *J* = 9.2 Hz, 2H), 3.27-3.19 (m, 4H), 2.92 (t, *J* = 8.8 Hz, 2H), 2.86 (d, *J* = 9.2 Hz, 2H), 2.70-2.64 (m, 4H), 2.52-2.13(m,

16H), 1.66 (t, $J = 3.2$ Hz, 2H), 1.54 (s, 6H), 1.47 (t, $J = 7.2$ Hz, 4H), 1.31-1.27 (m, 10H), 1.11 (t, $J = 11.6$ Hz, 2H) *ppm*. ^{13}C NMR (CDCl_3 , 100 MHz): δ 172.9, 171.1, 169.6, 138.8, 134.9, 130.4, 120.5, 81.2, 67.1, 63.4, 60.1, 42.8, 39.7, 36.7, 30.8, 29.6, 29.3, 29.1, 26.8, 26.2, 25.8, 24.5, 23.9, 18.1 *ppm*. HRMS (ESI) m/z calcd for $\text{C}_{47}\text{H}_{67}\text{N}_2\text{O}_{12}$ ($\text{M} + \text{H}$)⁺ 851.4689, found 851.4691.

7.2.8. bis-(((1*aR*,7*aS*,10*aS*,10*bS*,*E*)-1*a*-Methyl-8-methylene-9-oxo-1*a*,2,3,6,7,7*a*,8,9,10*a*,10*b*decahydrooxireno[2',3':9,10]cyclodeca[1,2-*b*]furan-5-yl)methyl)-4,4'-(decane-1,10-diyl bis-(azanediyl)) bis-(4-oxobutanoate) (4h)— ^1H NMR (CDCl_3 , 400 MHz): δ 6.25 (d, $J = 3.2$ Hz, 2H), 5.67 (t, $J = 7.2$ Hz, 2H), 5.60 (d, $J = 2.8$ Hz, 4H), 4.68 (d, $J = 12.8$ Hz, 2H), 4.49 (d, $J = 12.8$ Hz, 2H), 3.85 (t, $J = 8.8$ Hz, 2H), 3.25-3.21 (m, 4H), 2.91 (t, $J = 9.6$ Hz, 2H), 2.86 (d, $J = 9.2$ Hz, 2H), 2.69-2.64 (m, 4H), 2.52-2.09 (m, 16H), 1.66 (t, $J = 11.2$ Hz, 2H), 1.54 (s, 6H), 1.48 (t, $J = 7.2$ Hz, 4H), 1.31-1.27 (m, 12H), 1.11 (t, $J = 11.6$ Hz, 2H) *ppm*. ^{13}C NMR (CDCl_3 , 100 MHz): δ 172.9, 171.1, 169.6, 138.8, 134.9, 130.5, 120.5, 81.2, 67.1, 63.3, 60.1, 42.8, 39.7, 36.7, 30.8, 29.6, 29.5, 29.4, 29.2, 26.9, 25.8, 24.5, 23.9, 18.1 *ppm*. HRMS (ESI) m/z calcd for $\text{C}_{48}\text{H}_{69}\text{N}_2\text{O}_{12}$ ($\text{M} + \text{H}$)⁺ 865.4845, found 865.4843.

7.3. Methodology for the in vitro 60 human cancer cell screening assay

The methodology for the anti-cancer activity screening assay was carried out at the National Cancer Institute via the reported literature procedure.²² RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine was used for growing the NCI-60 human tumor cells. Initially the tumor cells were inoculated into 96-well microtiter plates in 100 microliters of medium at plating densities starting from 5,000 to 40,000 cells per well. The range of cell numbers is dependent on the doubling time of the individual tumor cell lines. The plates were then incubated at 37°C for 24 hours prior to addition of the test compounds. After 24 h, two microtiter plates of each tumor cell line were fixed *in situ* with TCA. The optical density reading at this point represents the cell population for each tumor cell line at the time of compound addition (OD_{zero}). The MMB dimers were dissolved in DMSO at 400-fold concentration to the desired final maximum test concentration, and stored at -80°C. Then, an aliquot part of the frozen concentrate was thawed and diluted to 10⁻⁴ M concentration with medium containing 50 µg/ml gentamicin. A control sample with just DMSO was also prepared. Aliquots of 100 microliters of the different MMB dimer dilution controls were added to the appropriate microtiter wells containing 100 µl of medium, resulting in the required final drug concentrations of 10⁻⁵ M and 0 M (control). Once the MMB analogs were added, the microtiter plates were incubated for 48 hrs at 37 °C and 100% relative humidity.

Cold TCA was used to terminate the assay for adherent cells. Cells were fixed by the addition of 50 µl of cold 50 % (w/v) TCA and further incubated for 1 hr at 4°C. The supernatant was discarded, and the microtiter plates were splashed five times with water and airdried. Sulforhodamine B (SRB) solution (100 microliters) at 0.4 % (w/v) in 1 % CH_3COOH was added to each well, and plates were incubated for another 10 minutes at room temperature. After SRB staining, free SRB was removed by washing five times with 1 % acetic acid. Bound SRB stain was successively dissolved with 10 mM trizma base, and

the optical density was measured at a wavelength of 515 nm. The growth inhibitory or cytotoxicity effects of the MMB analogs in the above cellular assay was measured by determining percentage cell growth (PG) inhibition. Optical density (OD) measurements of SRB-derived color just before exposing the cells to the test compound (OD_{zero}) and after 48hrs exposure to the test compound (OD_{test}) or the control vehicle (OD_{ctrl}) were recorded.

7.4. Cytotoxicity Screening with M9-ENL1 cells by Flow Cytometry

M9-ENL cells were plated at a density of 10⁶ cells/mL in alpha-MEM culture media (Invitrogen) supplemented with 5% human plasma, 20% FBS, and the cytokines SCF, IL-3, IL-7, and FLT3 (PeproTech). Drugs were diluted from a DMSO stock into PBS such that the final concentration of DMSO did not exceed 0.5%. Evaluations were performed after 24 h of drug exposure using viability labeling with trypan blue dye, as well as flow cytometric analysis by labeling with Annexin V and propidium iodide or 7-aminoactinomycin D to delineate apoptotic cell populations and to identify the percentage of non-apoptotic cells, which was defined as the population of cells with negative staining for both labels. The percentage of non-apoptotic cells observed was normalized to that of the vehicle control, and dose-response curves were analyzed using GraphPad Prism software to determine LC₅₀ values. All analyses were conducted in triplicate. In all these studies, PTL and MMB were included as reference standards. As expected, relative cytotoxicity varied considerably as a function of the specific modifications made to the MMB scaffold. Some of the conjugates demonstrated greater cytotoxicity than either PTL or MMB (Fig. 2).

7.5 Molecular Modeling Analysis

The protein structure of Nuclear transcription factor IKK β was retrieved from the protein data bank (PDBID: 4KIK).²⁹ All the water molecules were deleted for the docking analysis. No water molecule was found near the active site of the IKK β conserved cysteine residue Cys-46. Protein structure was processed to perform the docking calculations using Protein Preparation Wizard tool of Schrodinger³³. For the preparation of the protein structure, firstly, hydrogen molecules were added to the 4KIK and correct bond orders were assigned along with the deletions of crystallographic water molecules. Hydrogen bonds present in the protein structure were optimized at pH7 and restrained minimization was performed utilizing the PROPKA tool using the OPLS_2005 force field in Schrodinger. For the restrained minimization, RMSD was set to 0.3Å. The c and n terminal end of IKK β were uncapped to prepare it for the further docking.

MMB analog molecules were prepared using the *LigPrep* tool in Maestro program of Schrodinger to prepare for the docking studies.

A Docking grid was generated using the Maestro program of the Schrodinger. The grid boxes were generated around the conserved cysteine site Cys-46 on chain A. The maximum length of the docked ligands was set to 20Å. Covalent docking was performed using Covalent Dock. The reactive residue Cys-46 in IKK β is then mutated to an alanine to avoid the side chain influence while performing the docking procedure. Ligands were docked at the active binding site using (Cys-46) Glide protocol by performing *in silico* Michel addition reactions; best docking poses were then identified. The side chain of the reactive residue

(Ala-46) was then restored to the original residue (Cys-46) and best ten docking poses with minimum docking energy for the reactions were identified. At the end of this *in silico* reaction a covalent bond is formed at Cys-46 with the respective MMB analog. Then, the bound ligand and the reactive residue are minimized at the docking site. All the poses from the ligand are then clustered, and the best pose with minimum prime energy is identified for the final optimization. Affinity score is then calculated to determine the probability of the ligand and receptor interaction, and finally, one docked pose with the minimum energy is reported in the final output.

After docking, each docked MMB analog and the IKK β molecule were analyzed to understand binding mode and docking pattern using Pymol³⁴ visualization software supported by Schrodinger. Docked structures were examined for the quality of the covalent bond, and the number of hydrogen bonds generated in interaction of MMB analogs and IKK β graphics were generated using Pymol³⁴ for this analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Anti-cancer sesquiterpene lactones and their derivatives: parthenolide (**A**), costunolide (**B**), helenalin (**C**) melampomagnolide B (MMB) (**D**), carbamate ester analogs of MMB (**E**), dimeric carbonate and carbamate esters of MMB (**F** and **G**), and the orally bioavailable MMB imidazole-carbamate analog **H**.

Figure 2. Apoptotic activity for representative MMB derivatives **3i**, **3h**, **4g** and **3j** against cultured M9-ENL1 acute myelogenous leukemia cells

Figure 3.

(A) (left) human IKK β (PDBID: 4KIK)²⁹ chain covalently docked with the succinamide derivatives of melampomagnolide B (**4g**, **4f**, **3i**, and **3h**). These succinamide derivatives form covalent interactions with the highly-conserved cysteine-46 residue of the *N*-terminal lobe (1–109) of human IKK β (right) Zoomed interaction of succinamide derivatives of MMB (**4g**, **4f**, **3i**, **3h**) with IKK β (B) Interaction of **4g** (orange) with human IKK β Compound **4g** forms a covalent interaction with Cys-46 and forms hydrogen bonding interactions with Asn-28, Pro-88, and Glu-49. (C) Interaction of **4f** with human IKK β Compound **4f** forms a covalent interaction with Cys-46 and forms hydrogen bonding interactions with Asn-28, Glu-49, Ser-51 and Arg-57. (D) Interaction of **3i** with human IKK β Compound **3i** forms a covalent interaction with Cys-46 and forms a hydrogen bonding interaction with Glu-49. (E)

Interaction of **3h** with human IKK β . Compound **3h** forms a covalent interaction with Cys-46; however, it does not participate in any hydrogen bonding interactions.

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Scheme 1.
Synthesis of succinamide derivatives of MMB

Scheme 2.
Synthesis of succinamide dimers of MMB

Table 1

Structures, yields, reaction times, and melting points of succinamide analogs of MMB

Amine	Product	Yield (%)	Time (hr)	Mp (°C)
3a	75	6	oil
3b	72	5	oil
3c	65	8	oil
3d	85	12	175–177
3e	75	6	90–92

Amine	Product	Yield (%)	Time (hr)	Mp (°C)
3f	89	8	70–72
3g	70	6	oil
3h	65	12	150–151
3i	67	6	190–191
3j	71	3	120–122
3k	63	12	195–197

Amine	Product	Yield (%)	Time (hr)	Mp (°C)
3l	60	12	90–91

Table 2

Structures, yields, reaction times, and melting points of succinamido dimers of MMB

Product	Yield (%)	Time (hr)	Mp (°C)
<p>4a</p>	50	12	99–100
<p>4b</p>	57	12	96–97
<p>4c</p>	65	8	81–83
<p>4d</p>	65	6	95–96
<p>4e</p>	67	5	80–82

Product	Yield (%)	Time (hr)	Mp (°C)
4f	68	6	86–88
4g	70	6	76–77
4h	67	6	230–233

Table 3

Growth inhibition ($GI_{50}/\mu M$) data for MMB succinamide derivatives **4f**, **4g**, **3d** and **3i** against a panel of human cancer cell lines

Panel/cell line	4f GI_{50}	4g GI_{50}	3d GI_{50}	3i GI_{50}
Leukemia				
CCRF-CEM	0.32	0.28	2.20	1.40
HL-60(TB)	0.63	0.44	2.12	3.16
K-562	0.97	0.45	ND	3.06
MOLT-4	2.11	0.64	6.22	3.33
RPMI-8226	1.67	0.50	4.16	4.74
SR	0.41	0.33	2.59	2.72
Non-Small Cell Lung Cancer				
A549/ATCC	18.5	13.8	16.7	15.8
HOP-92	1.58	1.40	2.72	2.05
NCI-H226	2.06	1.90	12.7	3.51
NCI-H23	2.99	2.23	18.4	5.24
NCI-H322M	16.3	16.3	29.6	17.6
NCI-H460	5.0	2.18	21.3	16.0
NCI-H522	0.48	0.42	1.60	1.50
Colon cancer				
COLO 205	1.35	1.22	3.30	2.94
HCC-2998	4.12	2.25	14.4	11.5
HCT-116	1.54	0.86	3.62	1.69
HCT-15	3.60	2.63	3.43	1.70
HT29	1.64	1.33	ND	4.28
KM12	9.46	4.60	17.7	12.1
SW-620	1.22	0.54	4.35	2.33
CNS Cancer				
SF-268	3.34	2.92	10.5	13.2
SF-295	15.1	7.68	16.6	14.7
SF-539	2.43	1.85	3.25	2.35
SNB-19	4.15	3.38	15.6	14.0
SNB-75	2.02	1.47	4.59	11.2
U251	3.03	3.01	16.7	16.7
Melanoma				
LOX IMVI	1.37	0.37	4.74	2.95
MALME-3M	2.04	1.94	ND	2.95
M14	1.79	1.65	5.75	3.44
MDA-MB-435	1.80	1.63	4.95	4.84
SK-MEL-2	2.25	2.12	11.3	13.1
SK-MEL-28	1.65	1.47	3.44	3.10

Panel/cell line	4f GI ₅₀	4g GI ₅₀	3d GI ₅₀	3i GI ₅₀
SK-MEL-5	3.13	1.88	10.5	7.17
UACC-257	1.76	2.06	8.82	3.57
UACC-62	1.97	1.69	6.61	5.15
Ovarian Cancer				
OVCAR-3	1.50	1.29	4.54	4.32
OVCAR-4	1.64	1.48	10.1	2.64
OVCAR-5	1.82	1.70	12.1	6.36
OVCAR-8	2.90	3.24	12.5	3.37
NCI/ADR-RES	33.5	26.7	31.1	13.1
SK-OV-3	6.45	5.80	28.0	26.8
Renal Cancer				
786-0	1.62	1.49	4.43	6.64
A498	6.72	2.72	13.9	2.77
ACHN	3.13	2.10	4.45	2.64
CAKI-1	12.7	10.5	6.37	2.83
RXF 393	0.85	1.01	3.57	1.91
SN12C	3.74	2.69	12.5	4.80
TK-10	20.8	1.78	3.83	3.90
UO-31	10.9	3.90	10.2	3.56
Prostate Cancer				
PC-3	3.64	2.53	15.6	13.0
DU-145	1.92	1.75	3.84	4.44
Breast Cancer				
MCF7	0.77	0.42	3.20	2.22
MDA-MB-231/ATCC	1.97	1.58	6.19	7.13
HS 578T	3.88	2.99	14.7	20.4
BT-549	1.23	0.66	5.14	1.82
T-47D	1.51	1.49	3.52	5.45
MDA-MD-468	1.33	0.98	1.54	1.06

ND = not determined; GI₅₀ values <1 μ M are bolded

Table 4LC₅₀ values of succinamide analogs of MMB against M9-ENL1 cells.

Compound	LC ₅₀ value (μM)
PTL	7.5
3a	20
3b	>20
3c	13.5
3d	14
3e	14
3f	13
3g	14
3h	5.2
3i	4.1
3j	8.1
3k	60
3l	12
4a	10
4b	19
4c	19
4d	21
4e	15
4f	11
4g	6.5
4h	12

Table 5Molecular modeling interactions of succinamide derivatives of MMB with human IKK β

Compound	Covalent bonding interactions	Bond length	Docking score	Hydrogen bonding interaction
4g	Cys-46	4.207	-3.489	Asn-28
				Pro-88
				Glu-49
4f	Cys-46	4.746	-3.377	Asn-28
				Glu-49
				Ser-51
				Arg-57
3i	Cys-46	4.338	-2.426	Glu-49
3h	Cys-46	4.507	-1.643	