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Total Synthesis and Conformational Analysis of Naturally Occurring Lipovelutibols along with Lead Optimization of Lipovelutibol D

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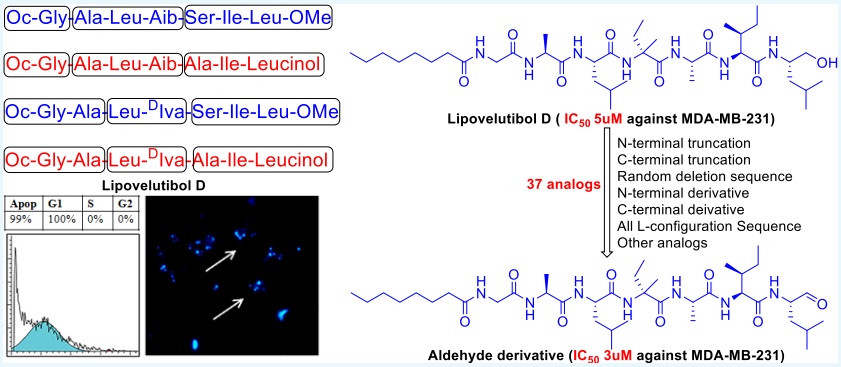

ABSTRACT: Four lipopeptaibols, namely, lipovelutibols A−D, were recently isolated from psychrotrophic fungus *Trichoderma velutinum* and reported to have significant cytotoxic activity against HL-60, MDA-MD-231, A549, and LS180 cancer cell lines. In the present study, these peptides were synthesized in a solution using a segment condensation approach. The conformational analysis of these peptides carried out using CD spectrophotometry revealed the formation of 3_{10}-helix, and the NMR-VT experiments showed intramolecular hydrogen bonding for NH-5, NH-6, and NH-7. Lipovelutibol D showed potent cytotoxic activity and was chosen for lead optimization. It involved N- and C-terminal truncation, N- and C-terminal modification, random deletion, L/D configuration replacement, and other synthetic analogues. These were tested against various breast cancer cell lines. The C-terminal aldehyde analogue resulting from lead optimization of lipovelutibol D was found to have almost twofold enhanced cytotoxicity against MDA-MB-231 breast cancer cell lines.

INTRODUCTION

Lipopeptaibols are a class of peptides that are similar to peptaibols and differ mainly by the presence of fatty acyl moieties of 8−15 carbon chains instead of acetyl moieties at N-termini. Other structural characteristics are similar to those of peptaibols such as the presence of one or more unusual α, α-dialkylated amino acids like α-amino isobutyric acid (Aib), ethylnorvaline, isovaline (Iva), and the C-terminal modified as amino alcohol.[1−4] These lipopeptaibols exhibit a wide range of activities, which mainly include antibacterial,[5] antifungal,[6] cytotoxic,[7] and such properties. The destabilization of the bilayer membrane caused by the lipopeptaibols attributes to their cytotoxic activity.[4] The chemical synthesis of these molecules is challenging due to the presence of sterically hindered α,α-dialkylated amino acids and acid-labile Aib-proline bonds and also the epimerization caused by Aib residues. Various synthetic strategies were adopted to overcome these problems. The azirine/oxazolone method,[8] the amino acid fluoride approach,[9] and microwave synthesis[10] are used to overcome these problems in the solid-phase peptide synthesis approach. However, in solution-phase peptide synthesis approaches, the segment was chosen in such a way to keep the Aib/Iva residue at the C-terminus to avoid epimerization, proline (Pro) was kept at the N-terminus of the fragment to avoid reaction between acid-labile Aib−Pro bonds,[11] the azirine/oxazolone method was used to introduce Aib−Pro sequence[12] and Aib/Iva units into the peptide chain,[13] and the symmetrical anhydride method to incorporate Aib[14] was used to overcome the problem. Out of vast peptaibols and lipopeptaibols available naturally, very few of them were synthesized in the solution phase, which includes hypomurocin A1,[12] trikoningin KB II,[14] emerimisin III and IV,[15] cervinin,[11] alamethicin F50/5,[16] zervamicin IIB,[17]

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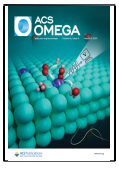


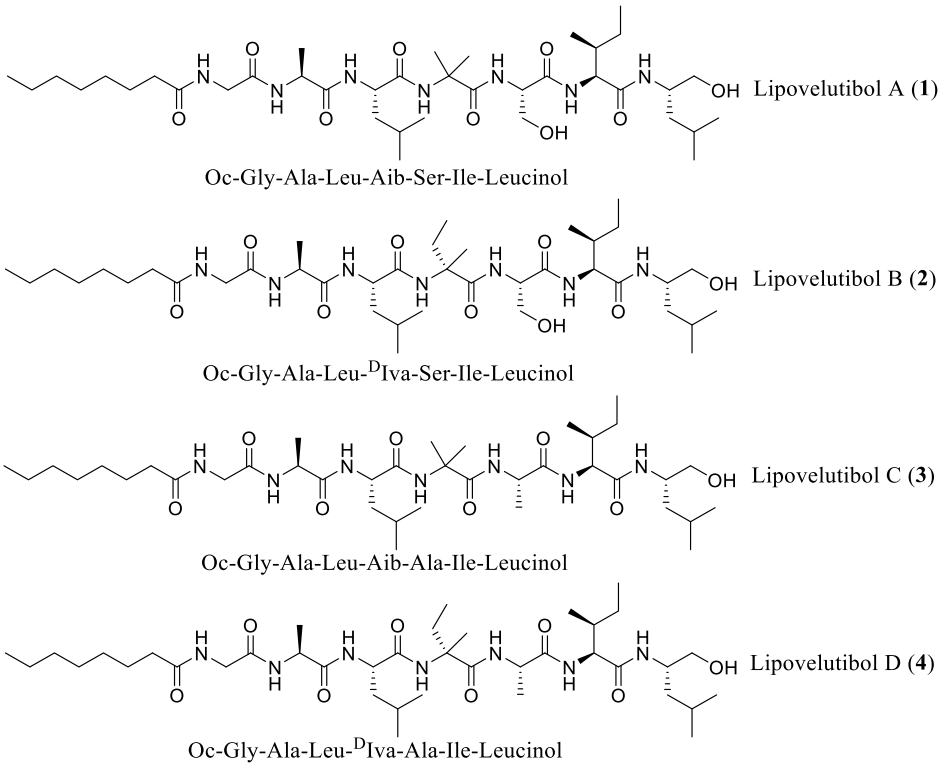
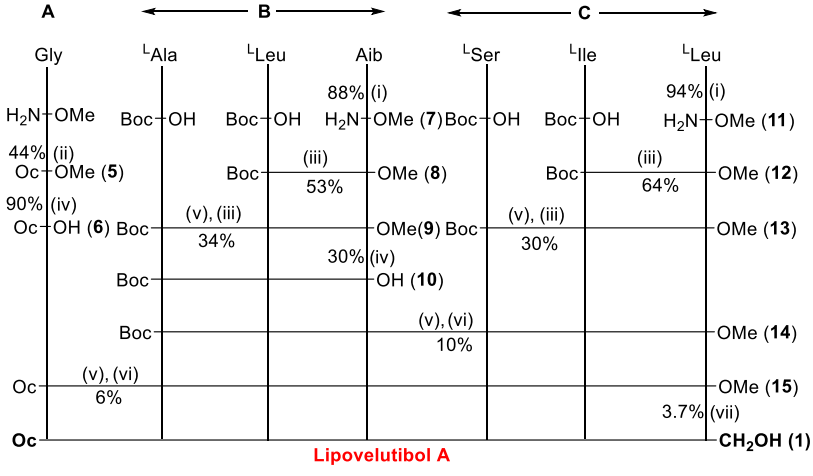


Figure 1. Structures of lipovelutibols A–D (**1**–**4**).

Scheme 1. Synthetic Scheme for the Synthesis of Lipovelutibol A (1)[a]



[a]Reagents and conditions: (i) MeOH, $SOCl_2$, 0 °C to rt, 4–6 h; (ii) octanoic acid,*N*-methylmorpholine (NMM), anhydrous dichloromethane (DCM), EDC.HCl, 6 h, rt; (iii) NMM, anhydrous DCM, EDC.HCl, 1-hydroxybenzotriazole (HOBt), 6–8 h, 0 °C to rt; (iv) NaOH (2 N), MeOH, 5–8 h, rt; (v) 25% trifluoroacetic acid (TFA) in DCM, 2 h, rt; (vi) NMM, anhydrous DCM, HBTU, HOBt, 24 h, 0 °C to rt; (vii) anhydrous tetrahydrofuran (THF), $LiBH_4$, 12 h, 0 °C to rt.

trichotoxin A-50 (G),[13] harzianin HB-I,[18] and alamethicin F-30.[19] The peptide molecules as drug candidate face various challenges like enzymatic hydrolysis, short half-life, fast renal clearance, difficulty in delivery via the oral route, and high molecular size. The chemical lead optimization of peptides becomes a crucial tool to overcome these problems. Utilization of a combinatorial optimization strategy,[20] delivery of peptides through liposomes and protein transduction domains,[21] a quantitative structure–activity relationship (QSAR) approach including systemic amino acid substitution[22] are used for the purpose of lead optimization. Chemical lead optimization approaches, like truncation from the N- or C-terminal;[23] alanine scanning and D-scanning;[23] macrocyclization;[23,24] natural amino acid replacement with an unnatural amino acid residue;[23] N- or C-terminal modification;[23,2423,24] *N*-methylation;[25] introducing intramolecular hydrogen bonds; and using biotin conjugation,[25] vitamin B12 uptake systems,[25] absorption enhancers,[25] and conjugating peptides with large synthetic or natural polymers,[25] were widely used and suggested for peptide lead optimization.

The presence of nonproteogenic amino acids like Aib or Iva and modified N- and C-terminus in peptaibol and lip-

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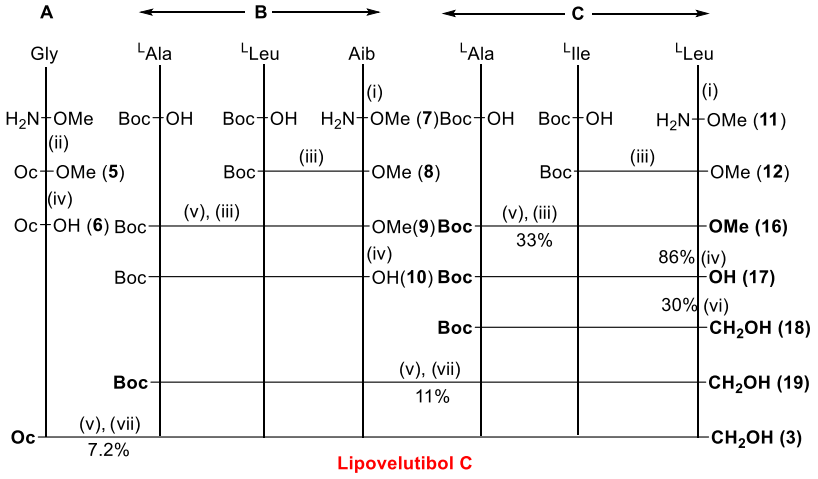
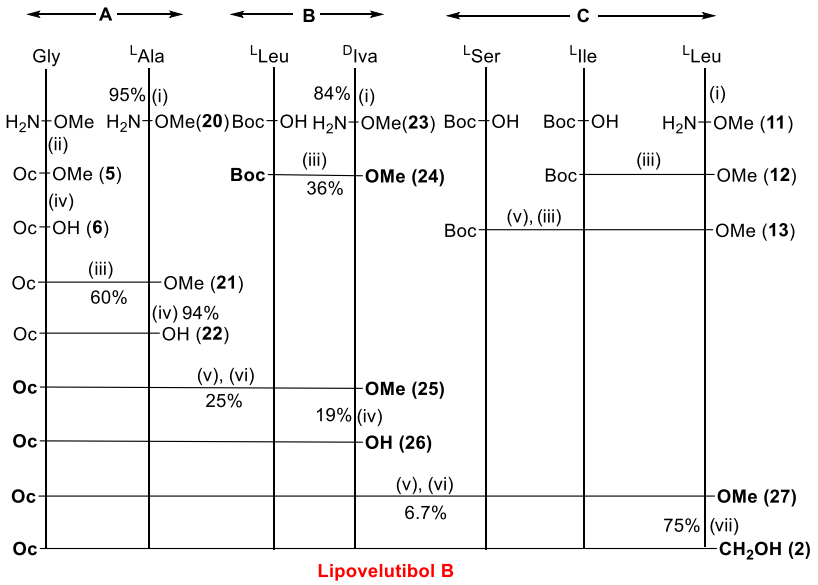
Scheme 2. Synthetic Scheme for the Synthesis of Lipovelutibol C (3)[a]



[a]Reagents and conditions: (i) MeOH, $SOCl_2$, 0 °C to rt, 4−6 h; (ii) octanoic acid, NMM, anhydrous DCM, EDC.HCl, 6 h, rt; (iii) NMM, anhydrous DCM, EDC.HCl, HOBt, 6−8 h, 0 °C to rt; (iv) NaOH (2 N), MeOH, 5−8 h, rt; (v) 25% TFA in DCM, 2 h, rt; (vi) dry THF, DIC, aqueous $NaBH_4$, 2 h, 0 °C to rt; (vii) NMM, anhydrous DCM, HBTU, HOBt, 24 h, 0 °C to rt.

Scheme 3. Synthetic Scheme for the Synthesis of Lipovelutibol B (2)[a]



[a]Reagents and conditions: (i) MeOH, $SOCl_2$, 0 °C to rt, 4−6 h; (ii) octanoic acid, NMM, anhydrous DCM, EDC.HCl, 6 h, rt; (iii) NMM, anhydrous DCM, EDC.HCl, HOBt, 6−8 h, 0 °C to rt; (iv) NaOH (2 N), MeOH, 5−8 h, rt; (v) 25% TFA in DCM, 2 h, rt; (vi) NMM, anhydrous DCM, HBTU, HOBt, 24 h, 0 °C to rt; (vii) anhydrous THF, $LiBH_4$, 12 h, 0 °C to rt.

opeptaibols imparts certain advantages over other natural peptides like greater plasma stability, disruption in the membrane potential, and easy permeability through the biological membrane. Thus, these peptaibiotics can be advantageous in lead optimization and drug discovery program.

We recently isolated four lipopeptaibols i.e., lipovelutibols A−D (**1**−**4**),[26] from fungus *Trichoderma velutinum*, as shown in Figure 1. To confiscate dependency on microbial production and have the compounds in quantity for further analysis, we herein carry out the total synthesis of these lipovelutibols through the segment condensation approach via solution-phase peptide synthesis. This is the first report of total synthesis of all of the lipovelutibols via peptide synthetic approaches in solution, although recently two of the lipovelutibols (B and D) have been synthesized via solid-phase peptide synthesis (SPPS).[27] These peptides were further studied for their conformational analysis and the hydrogen-bonding pattern. As evident from the previous study that compound **4** showed potent cytotoxicity against the cell lines tested, lead optimization of **4** via N- and C-terminal truncation, N- and C-terminal modification, random deletion, L/D configuration replacement, and other synthetic analogues was also carried out. We also carried out cell cycle analysis for **4** against HL-60 cell lines to determine apoptosis and phase distribution.

RESULTS AND DISCUSSION

Total Synthesis of Lipovelutibol A (1), B (2), C (3), and D (4). The lipovelutibols were synthesized via the segment condensation approach through solution-phase peptide syn-

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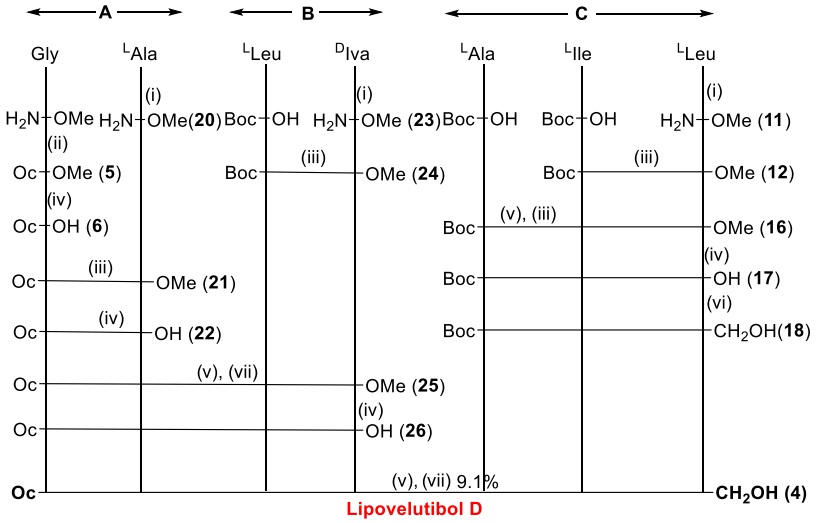
Scheme 4. Synthetic Scheme for the Synthesis of Lipovelutibol D (4)[a]



[a]Reagents and conditions: (i) MeOH, $SOCl_2$, 0 °C to rt, 4−6 h; (ii) octanoic acid, NMM, anhydrous DCM, EDC.HCl, 6 h, rt; (iii) NMM, anhydrous DCM, EDC.HCl, HOBt, 6−8 h, 0 °C to rt; (iv) NaOH (2 N), MeOH, 5−8 h, rt; (v) 25% TFA in DCM, 2 h, rt; (vi) dry THF, DIC, aqueous $NaBH_4$, 2 h, 0 °C to rt; (vii) NMM, anhydrous DCM, HBTU, HOBt, 24 h, 0 °C to rt.

thesis. These peptides contain α,α dialkylated amino acids like Aib in their sequence; thus, the segment coupling can cause epimerization at the penultimate C-terminal residue by tautomerization of the oxazolone intermediate.[28] Two different synthetic strategies were adopted for four lipovelutibols: one for Aib containing lipovelutibols, i.e., [1 + (3 + 3)] for compounds **1** and **3** and another for lipovelutibols containing Iva residues, i.e., [(2 + 2) + 3] for compounds **2** and **4**, as shown in scheme 1−4. In both synthetic strategies, the Aib/Iva residue was kept at C-terminus to avoid the chance of epimerization.

Total Synthesis of Lipovelutibol A (1). *Synthesis of Fragments.* As discussed above, peptide **1** was synthesized through condensation of three fragments. Fragment A was synthesized by coupling glycine-methyl ester with octanoic acid using EDC.HCl to get Oc-Gly-OMe (**5**). Compound **5** was further hydrolyzed by aqueous NaOH (2M) to get Oc-Gly-OH (**6**) as fragment A. Tripeptide fragment B was synthesized by step-by-step coupling of three protected amino acids. First, the methyl ester of amino acid Aib, i.e., Aib-OMe (**7**), was synthesized. Further, compound **7** was coupled with Boc-leucine using EDC.HCl/HOBt as a coupling agent to obtain dipeptide methyl ester Boc-Leu-Aib-OMe (**8**). The resulting dipeptide was deprotected using 25% TFA in DCM and then coupled with freshly activated Boc-alanine with EDC.HCl/HOBt to get the tripeptide methyl ester Boc-Ala-Leu-Aib-OMe (**9**). It was then hydrolyzed to get compound Boc-Ala-Leu-Aib-OH (**10**) as fragment B. For fragment C, compound Boc-isoleucine was coupled with previously synthesized Leu-OMe (**11**) to get dipeptide Boc-Ile-Leu-OMe (**12**). This was subjected to Boc-deprotection and subsequently coupling to Boc-serine using EDC/HOBt as a coupling agent to get Boc-Ser-Ile-Leu-OMe (**13**). The reduction of this peptide to tripeptide alcohol incurred yield loss. So, the reduction was planned to be carried out at the final stage and compound **13** was taken as fragment C for further coupling (Scheme 1 and Scheme S1 in the Supporting Information).

Segment Condensation. Compound **10** (fragment B) was coupled with Boc-deprotected **13** (fragment C) using HBTU/HOBt as a coupling agent and N-methylmorpholine (NMM) as a base in anhydrous dichloromethane (DCM) to obtain hexapeptide methyl ester Boc-Ala-Leu-Aib-Ser-Ile-Leu-OMe (**14**). Compound **14** was deprotected and coupled with compound **6** (fragment A) using the HBTU/HOBt coupling strategy to obtain **15**, a methyl ester precursor of **1**. Upon reduction of compound **15** with $LiBH_4$, the putative lipopeptaibol **1** was obtained in overall 12 steps (Scheme 1 and Scheme S2 in the Supporting Information).

Total Synthesis of Lipovelutibol C (3). Compound **3** was synthesized using a similar fragmentation approach to that of **1** (Scheme 1). For fragment C, the tripeptide Boc-Ala-Ile-Leu-OMe (**16**) was synthesized via coupling between dipeptide **12** and Boc-alanine. The resulting tripeptide was hydrolyzed to Boc-Ala-Ile-Leu-OH (**17**) followed by reduction using aqueous $NaBH_4$ to get Boc-Ala-Ile-Leu-CH_2OH (**18**) (fragment C). Further, a similar strategy of {1 + (3 + 3)} was adopted to get **3** in 13 steps (Scheme 2, Scheme S3 Supporting Information).

Total Synthesis of Lipovelutibol B (2). Here, the tripeptide Boc-Ser-Ile-Leu-OMe was taken as fragment C, which was previously synthesized and utilized in the total synthesis of **1**. Herein, we initially tried a similar tripeptide strategy (fragment B in the synthesis of **1** and **3**) but incurred yield loss while coupling Boc-alanine with the dipeptide. More importantly, it is important to mention here that amino acid ^{D}Iva was costly and available in a very small quantity. Owing to the cost and availability of ^{D}Iva, the synthesis strategy was changed to (A + B) + C, i.e., (2 + 2) + 3 coupling scheme, as shown in Scheme 3. Fragment A was synthesized by coupling **6** with previously synthesized Ala-OMe (**20**) to get dipeptide Oc-Gly-Ala-OMe (**21**). Compound **21** was subsequently hydrolyzed using aqueous NaOH in methanol to get Oc-Gly-Ala-OH (**22**) (fragment A). The fragment B dipeptide was synthesized by coupling commercially available Boc-leucine with the previously synthesized ^{D}Iva-OMe (**23**) to get Boc-Leu-^{D}Iva-OMe (**24**) (fragment B). Fragment B was then

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deprotected to get free N-terminus and subsequently coupled with **22** using HBTU/HOBt as a coupling agent to get tetrapeptide Oc-Gly-Ala-Leu-^{D}Iva-OMe (**25**). Compound **25** was further hydrolyzed with aqueous NaOH (2N) to obtain Oc-Gly-Ala-Leu-^{D}Iva-OH (**26**). Compound **26** was coupled with freshly deprotected **13** using the HBTU/HOBt coupling strategy to get heptapeptide Oc-Gly-Ala-Leu-^{D}Iva-Ser-Ile-Leu-OMe (**27**), a methyl ester precursor of compound **2**. The resulting heptapeptide was then reduced by $LiBH_4$ in anhydrous THF to give **2** in 14 synthetic steps (Scheme 3 and Scheme S4 in the Supporting Information).

Total Synthesis of Lipovelutibol D (4). For the synthesis of **4**, a similar synthetic strategy of (2 + 2) + 3 was utilized. Herein, the fragments synthesized earlier, i.e., compounds **26** (tetrapeptide) and **18** (tripeptide alcohol), were coupled using HBTU/HOBt as the coupling agent. This led to the synthesis of lipopeptaibol **4** as shown in Scheme 4 (Scheme S5 Supporting Information) in a total of 15 steps.

The ^{1}H, ^{13}C NMR, and high-resolution mass spectrometry (HRMS) spectra along with the high-performance liquid chromatography (HPLC) chromatograms for the synthetic lipopeptaibols were matched and found identical with the natural lipopeptaibols.[26] A comparison of the HPLC chromatograms of natural and synthetic lipovelutibols is given in Figure 2.

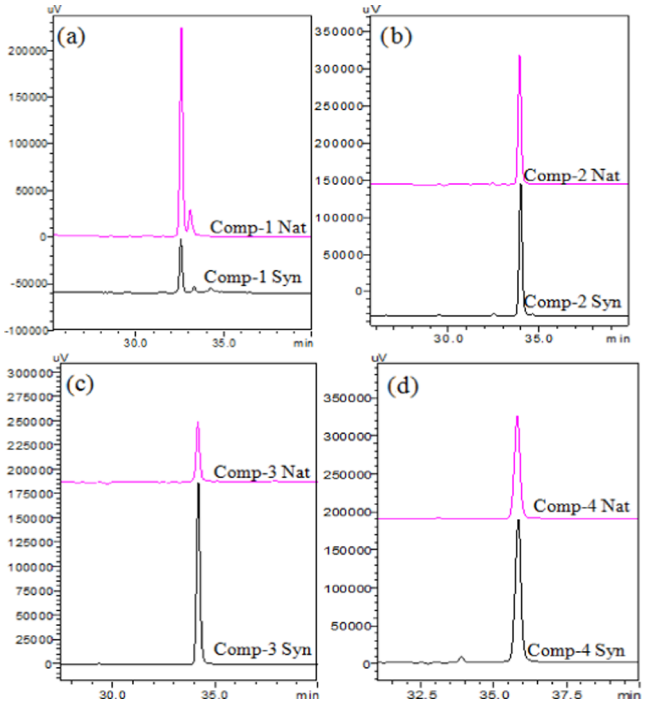

Figure 2. HPLC chromatograms of natural and synthetic lipopeptaibols **1**, **2**, **3**, and **4**.

Conformational Analysis of Lipovelutibol A (1), B (2), C (3), and D (4). A conformational study was performed through the CD technique[29,30] and the hydrogen-bonding pattern was established by NMR-VT (variable-temperature)[31,32] experiments. The CD spectra of compounds **1**, **2**, **3**, and **4** (both natural and synthetic) in methanol were analyzed (Figure S188, Supporting Information). The CD spectrum of **1** showed a negative maxima at 203 nm and a positive shoulder in the vicinity of 222 nm, i.e., at 220 nm. The conformationally sensitive ellipticity ratio R $[\theta_T^{220}]/[\theta_T^{203}]$ was found to be 0.16 for natural and 0.17 for synthetic compound **1**. Similarly, in the case of compound **2**, the ellipticity ratio R $[\theta_T^{224}]/[\theta_T^{204}]$ was found to be 0.07 for natural and 0.11 for the synthetic form. The ellipticity ratio R $[\theta_T^{220}]/[\theta_T^{204}]$ for compound **3** was found to be 0.25 for natural and 0.22 for its synthetic form and was 0.25 for natural and 0.18 for the synthetic form of **4**. These values of R clearly indicated that compounds **1**, **2**, **3**, and **4** fold in a 3_{10}-helix in methanol as the value for R is known to be less than 0.50 for a high population of 3_{10}-helix.

The NMR-VT study examined the proton NMR spectra of the NH region and its behavior as a function of temperature.[31,32] ^{1}H NMR spectra of each lipopeptaibol were recorded at four different temperatures, i.e., 298, 308, 318, and 328 K, in dimethyl sulfoxide (DMSO)-d_6. The chemical shifts (δ values of ^{1}H NMR) of NH-1 to NH-4 showed a significant shift with an increase in temperature, whereas NH-5, NH-6, and NH-7 had very little change in the chemical shifts for lipopeptaibols **1**, **2**, **3**, and **4**, as shown in Figure 3 (Figures

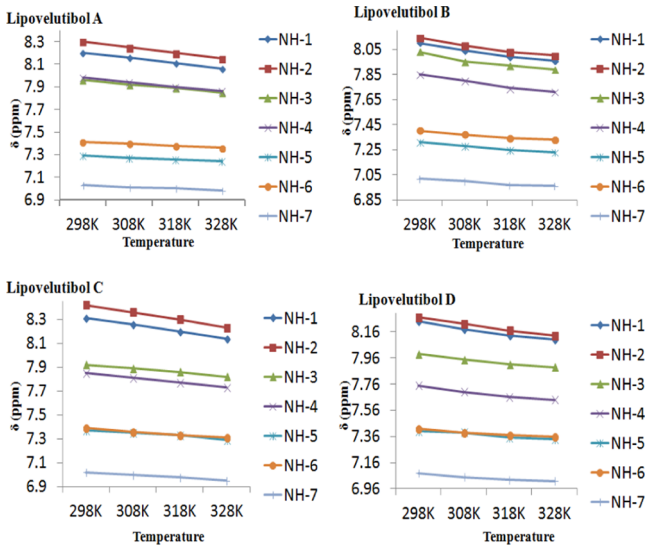

Figure 3. NMR-VT experiment plot of NH chemical shifts in the ^{1}H NMR spectra carried out at four different temperatures for lipovelutibols A (**1**), B (**2**), C (**3**), and D (**4**).

S189–S192, Supporting Information). Thus, it was evident from the NMR-VT experiment that three of NH protons, i.e., NH-5, NH-6, and NH-7, were involved in intramolecular hydrogen bonding in all of the four putative peptides.

Combining both the above experiments, it was inferred that these peptides fold in the 3_{10}-helical conformation, wherein NH-5, NH-6, and NH-7 are involved in (i/i + 3) hydrogen-bonding pattern with the corresponding carbonyl moiety.

Lead Optimization of Lipovelutibol D (4). As discussed previously, the MTT assay of compound **4** exhibited inhibitory activity against cancer cell lines, viz., MDA-MD-231, A549, LS180, and HL-60 with IC_{50} values of 5, 4, 7, and 4 μM, respectively.

It was also evident from the previous report that only compound **4** showed cytotoxicity against all of the cell lines tested.[26] Thus, peptide **4** was taken as the lead peptide molecule and optimized to improve its potency. Herein, all of the peptide derivatives were synthesized via the solution-phase strategy.

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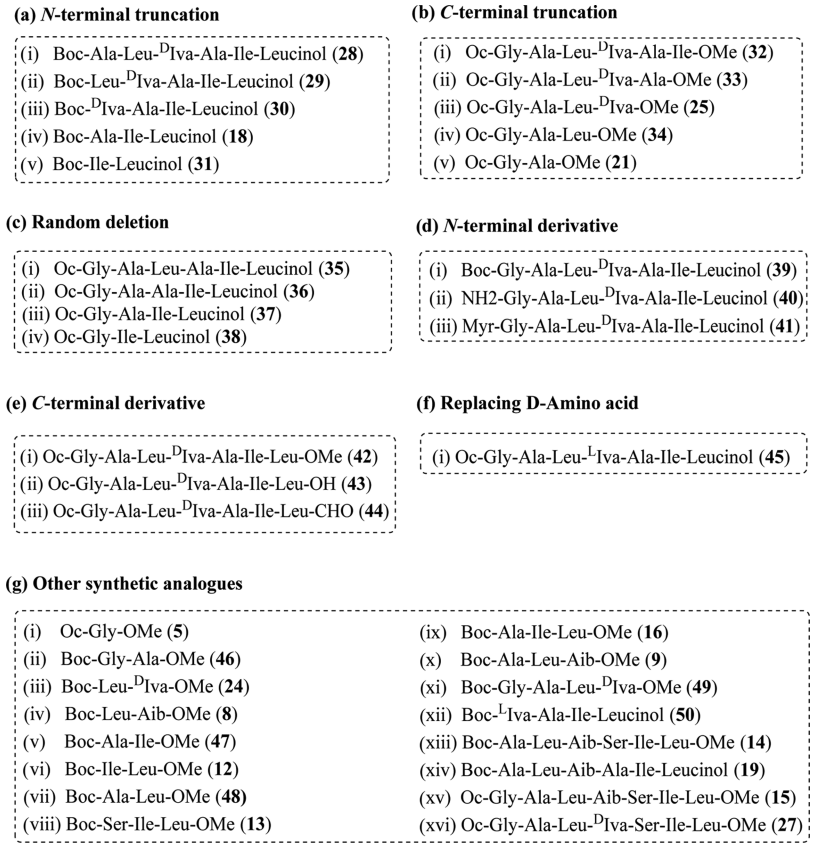

Figure 4. Lead optimization of **4**.

N- and C-Terminal Truncated Analogues. The N- and C-terminal truncation was carried out for the putative peptide as a strategy to know the role of amino acids in and to determine the minimum active sequence for cytotoxic activity. Truncation from N-terminus gave five peptide derivatives, i.e., Boc-Ala-Leu-^{D}Iva-Ala-Ile-Leucinol (**28**), Boc-Leu-^{D}Iva-Ala-Ile-Leucinol (**29**), Boc-^{D}Iva-Ala-Ile-Leucinol (**30**), **11** and Boc-Ile-Leucinol (**31**), as shown in Figure 4a. Similarly, truncation from C-terminus led to the synthesis of five peptide derivatives, i.e., Oc-Gly-Ala-Leu-^{D}Iva-Ala-Ile-OMe (**32**), Oc-Gly-Ala-Leu-^{D}Iva-Ala-OMe (**33**), **25**, Oc-Gly-Ala-Leu-OMe (**34**), and **21**, as shown in Figure 4b.

Random Deletion Analogues. Peptide **4** was subjected to random deletion of one or more amino acid to determine the role of amino acids in and a shorter active sequence for cytotoxicity. A total of four peptides were designed and synthesized, which includes Oc-Gly-Ala-Leu-Ala-Ile-Leucinol (**35**), Oc-Gly-Ala-Ala-Ile-Leucinol (**36**), Oc-Gly-Ala-Ile-Leucinol (**37**), and Oc-Gly-Ile-Leucinol (**38**). Hexapeptide **35** was intended to remove ^{D}Iva from the molecule as one of the strategies to know its role in the cytotoxicity of **4**. Here, all of the deletions were made on and around ^{D}Iva (Figure 4c).

N- and C-Terminal Derivatives. The derivatives of compound **4** with modification at the N- and C-terminus were designed to have a change in hydrophobicity of the putative peptide and to establish their effect on cytotoxicity. Herein, three peptides each with N- and C-terminal modifications were synthesized. This includes Boc-Gly-Ala-Leu-^{D}Iva-Ala-Ile-Leucinol (**39**), NH_2-Gly-Ala-Leu-^{D}Iva-Ala-Ile-Leucinol (**40**), and Myr-Gly-Ala-Leu-^{D}Iva-Ala-Ile-Leucinol (**41**) for N-terminal modification and Oc-Gly-Ala-Leu-^{D}Iva-Ala-Ile-Leu-OMe (**42**), Oc-Gly-Ala-Leu-^{D}Iva-Ala-Ile-Leu-OH (**43**), and Oc-Gly-Ala-Leu-^{D}Iva-Ala-Ile-Leu-CHO (**44**) as C-terminal-modified peptides (Figure 4d,e).

Replacement of ^{D}Iva with ^{L}Iva. To examine the cytotoxic effect of ^{D}Iva in the sequence, an analogue with all L-amino acids, i.e., Oc-Gly-Ala-Leu-^{L}Iva-Ala-Ile-Leucinol (**45**), was designed and synthesized (Figure 4f).

Other Synthetic Intermediates and Analogues. Herein, all of the other synthetic intermediates from the synthesis of **1**, **2**, **3**, and **4** are tested for cytotoxicity to know about the minimum active sequence (Figure 4g).

Biological Evaluation of All of the Analogues and Derivatives. The MTT assay was performed to examine the effect of compounds on the proliferation of cancer cell lines. Three breast cancer cell lines, viz., MDA-MB-231, T47D, and MCF-7, are treated with each of the compounds with a single concentration of 10 μM. The compounds having more than 50% growth inhibition were considered active (shown as bold entries in Table 1). The cytotoxicity of all of the above compounds was compared against **4** (Table 1).

The results revealed that for the C-terminus derivatives compounds like methyl ester (**42**) and C-terminal aldehyde (**44**) were active against MDA-MB-231 cancer cells having 68% and 77% growth inhibition, respectively, whereas the C-terminus COOH derivative (**43**) was found inactive. However, at N-terminus, compound **41**, the myristoyl chain analogue, was also active against MDA-MB-231 cancer cell lines and showed 82% growth inhibition. None of the smaller sequence analogues have shown activity against MDA-MB-231 cancer cell lines.

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Table 1. Preliminary Screening of Compounds Showing % Growth Inhibition of Human Breast Cancer Cell Lines at 10 μM

s. no.	compounds	human breast cancer cell lines (% growth inhibition)		
		MDA-MB 231	T47D	MCF-7
1	Oc-Gly-OMe (**5**)	10	23	45
2	Boc-Leu-Aib-OMe (**8**)	20	12	28
3	Boc-Ala-Leu-Aib-OMe (**9**)	7	3	43
4	Boc-Ile-Leu-OMe (**12**)	12	28	30
5	Boc-Ser-Ile-Leu-OMe (**13**)	14	24	24
6	Boc-Ala-Leu-Aib-Ser-Ile-Leu-OMe (**14**)	27	0	21
7	Oc-Gly-Ala-Leu-Aib-Ser-Ile-Leu-OMe (**15**)	26	2	23
8	Boc-Ala-Ile-Leu-OMe (**16**)	17	13	29
9	Boc-Ala-Ile-Leucinol (**18**)	9	17	7
10	Boc-Ala-Leu-Aib-Ala-Ile-Leucinol (**19**)	22	11	0
11	Oc-Gly-Ala-OMe (**21**)	28	30	35
12	Boc-Leu-^{D}Iva-OMe (**24**)	25	12	28
13	Oc-Gly-Ala-Leu-^{D}Iva-OMe (**25**)	27	9	40
14	Oc-Gly-Ala-Leu-^{D}Iva-Ser-Ile-Leu-OMe (**27**)	20	0	26
15	Boc-Ala-Leu-^{D}Iva-Ala-Ile-Leucinol (**28**)	29	34	45
16	Boc-Leu-^{D}Iva-Ala-Ile-Leucinol (**29**)	34	28	48
17	Boc-^{D}Iva-Ala-Ile-Leucinol (**30**)	34	34	38
18	Boc-Ile-Leucinol (**31**)	25	26	32
19	Oc-Gly-Ala-Leu-^{D}Iva-Ala-Ile-OMe (**32**)	30	34	43
20	Oc-Gly-Ala-Leu-^{D}Iva-Ala-OMe (**33**)	31	35	46
21	Oc-Gly-Ala-Leu-OMe (**34**)	34	0	36
22	Oc-Gly-Ala-Leu-Ala-Ile-Leucinol (**35**)	33	25	47
23	Oc-Gly-Ala-Ala-Ile-Leucinol (**36**)	21	30	25
24	Oc-Gly-Ala-Ile-Leucinol (**37**)	10	22	38
25	Oc-Gly-Ile-Leucinol (**38**)	30	0	18
26	Boc-Gly-Ala-Leu-^{D}Iva-Ala-Ile-Leucinol (**39**)	13	16	45
27	NH_2-Gly-Ala-Leu-^{D}Iva-Ala-Ile-Leucinol (**40**)	31	34	**50**
28	Myr-Gly-Ala-Leu-^{D}Iva-Ala-Ile-Leucinol (**41**)	**82**	32	**59**
29	Oc-Gly-Ala-Leu-^{D}Iva-Ala-Ile-Leu-OMe (**42**)	**68**	43	**64**
30	Oc-Gly-Ala-Leu-^{D}Iva-Ala-Ile-Leu-OH (**43**)	19	35	49
31	Oc-Gly-Ala-Leu-^{D}Iva-Ala-Ile-Leucinal (**44**)	77	29	41
32	Oc-Gly-Ala-Leu-^{L}Iva-Ala-Ile-Leucinol (**45**)	41	37	46
33	Boc-Gly-Ala-OMe (**46**)	38	2	37
34	Boc-Ala-Ile-OMe (**47**)	21	0	7
35	Boc-Ala-Leu-OMe (**48**)	23	0	38
36	Boc-Gly-Ala-Leu-^{D}Iva-OMe (**49**)	25	22	**51**
37	Boc-^{L}Iva-Ala-Ile-Leucinol (**50**)	7	24	29
38	Lipovelutibol D (**4**)	**57**	**53**	**56**

Methyl ester analogue **42** was also active against MCF-7 cells with 64% growth inhibition at 10 μM concentration. Two analogues of the N-terminus derivative, i.e., compound **40**—the N-terminal free analogue—and compound **41**—the myristoyl chain analogue, are active against MCF-7 cells, showing 50% and 59% growth inhibition, respectively. Apart from these heptapeptide derivatives, tetrapeptide **49** was found to be active against MCF-7 cell lines with 51% growth inhibition. Apart from these, none of the other analogues has shown significant inhibition. Interestingly, none of the compounds other than **4** was active against T47D breast cancer cell lines.

The cytotoxic evaluation of compound **4** analogues against MDA-MB-231 and MCF-7 cancer cell lines also revealed that ^{D}Iva at position 4 is required for activity as it is evident from the cytotoxic assay of compound **45** (analogue with ^{L}Iva at position 4) and compound **35** (analogue without ^{D}Iva at position 4) having low % inhibition. Importantly, compound **4** has shown more than 50% growth inhibition against all of the three breast cancer cell lines. Cytotoxic evaluation of compound **4** and its analogues against MDA-MB-231 and MCF-7 cancer cell lines is depicted in Figure 5.

IC_{50} Estimation of Compounds. Compounds **41**, **42**, and **44** showing higher % growth inhibition than that of **4** was taken further for IC_{50} calculation against MDA-MB-231 cell lines. The IC_{50} values of these compounds were found to be 6 $\pm$ 1, 7 $\pm$ 2, and 3 $\pm$ 1 μM, respectively, whereas the value was 5 μM for compound **4** against the MDA-MB-231 cell line as reported earlier.[26] The IC_{50} experiment was done in triplicate. We herein observed an almost twofold increase in cytotoxicity for the C-terminal aldehyde analogue.

Cell Cycle Assay of 4. In another study, the cell cycle assay of compound **4** obtained by microbial isolation was carried out against a human myeloid leukemia cell line (HL-60). The effect of **4** on cell cycle phase distribution and cellular and nuclear morphology in human myeloid leukemia HL-60 was studied. Compound **4** showed 75, 77, and 99% apoptotic DNA population at 10, 20, and 30 μM, respectively (Figure 6a). Compound **4** showed apoptotic DNA damage in a dose-dependent manner. The apoptotic bodies are visualized under a fluorescence microscope using 4′,6-diamidino-2-phenylindole (DAPI) as a DNA staining dye. The small apoptotic bodies are indicated by white arrows in Figure 6b and also revealed the formation of apoptotic bodies increase in a dose-dependent manner. The nuclear morphology of the HL-60 cells was observed through phase-contrast microscopy. Bleeding and distortion were observed in the treated cells (Figure 6c). The control experiment for the cell cycle assay performed here was the same as that in our recently published work.[33]

CONCLUSIONS

All of the four lipovelutibols **1**, **2**, **3**, and **4** were synthesized via a segment condensation approach in solution. These peptides fold in the 3_{10}-helical conformation, where NH-5, NH-6, and NH-7 were involved in $(i/i + 3)$ intramolecular hydrogen bonding. Chemical lead optimization of peptide **4** resulted in the synthesis of 37 analogues, and cytotoxic assays against a variety of breast cancer cell lines (MDA-MB-231, T47D, and MCF-7) were carried out. Different analogues have shown activity against MDA-MB-231 and MCF-7, but none of the analogues other than compound **4** was found active against the T47D cell line. The IC_{50} calculation against MDA-MB-231 breast cancer cell lines revealed an almost twofold increase in cytotoxicity for the C-terminal aldehyde analogue as compared with **4**. The cell cycle assay of **4** revealed apoptotic DNA damage in HL-60 cells in a dose-dependent manner. The systemic medicinal chemistry approach will further aid in designing new analogues with better physiochemical properties.

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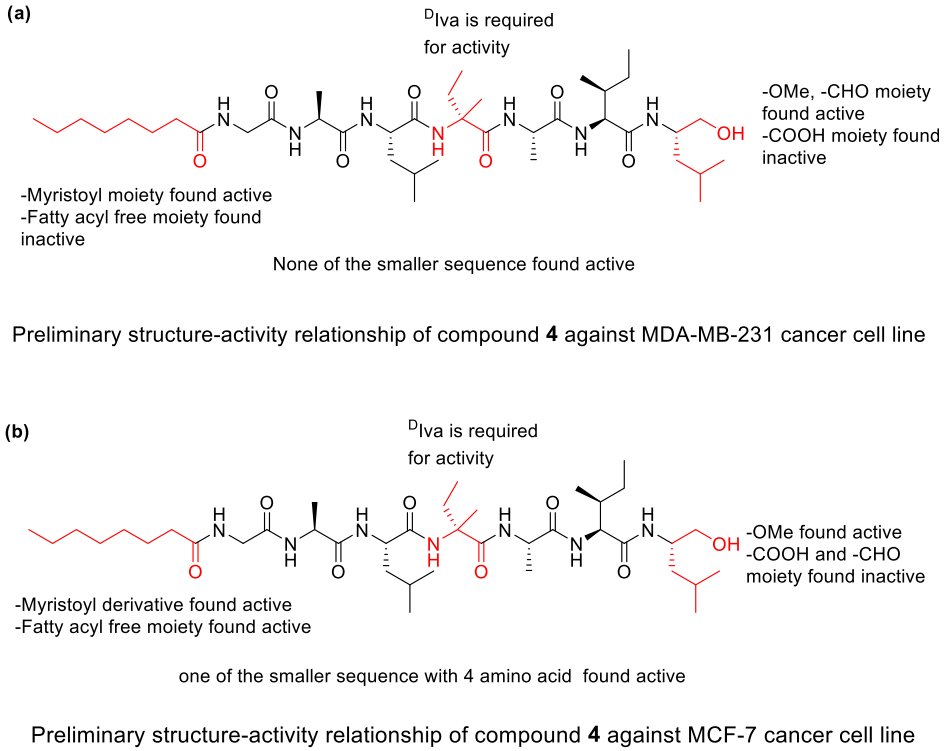

Figure 5. Preliminary structure−activity relationship of compound **4** against (a) MDA-MB-231 and (b) MCF-7 cancer cell lines.

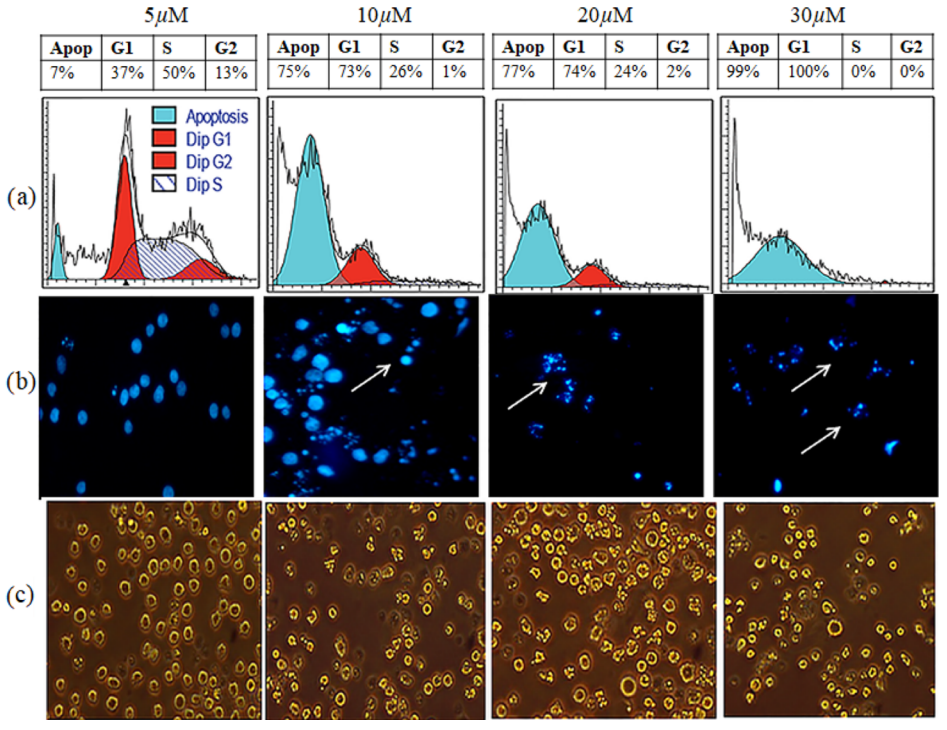

Figure 6. Effect of **4** (a) on cell cycle phase distribution, (b) on the morphology, and (c) on the nuclear morphology of HL-60 cells.

EXPERIMENTAL SECTION

General Experimental Procedure. The HPLC-grade solvents such as water, methanol, and acetonitrile were purchased from Merck, India. The amino acids such as ^{D}Iva, ^{L}Iva, and Boc-^{L}isovaline were purchased from Thermo Fisher Scientific. All of the other amino acids such as Aib, Boc-leucine, Boc-valine, alanine, serine, and glycine-methyl ester were purchased from Sigma-Aldrich, India. The chemicals such as propidium iodide (PI), 3-(4,5,-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RPMI-1640 media, and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich. The breast cancer cell lines MDA-MB -231, T47D, and MCF-7 were purchased from Sigma-Aldrich, India (ECACC), and the human myeloid leukemia cell line (HL-60) was purchased from ECACC, England.

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HRMS was performed on an Agilent 6540 UHD quadrupole time-of-flight (Q-ToF) mass spectrometer coupled with a 1260 affinity series LC system. The capillary voltage was kept at 3500 V, at temperature 350 °C with 7.0 L/min auxiliary gas flow. The spray voltage was kept at 4.5 kV, and a mass range of 100−1000 amu was selected with a maximum resolution of 30000 for HRMS analysis. NMR measurements including 1D proton, carbon, and DEPT-135 experiments were recorded on Bruker Avance III 400 and Bruker Avance DPX FT-NMR 500 MHz spectrometers. The NMR-VT experiment was performed on a thermostat-enabled Bruker Avance DPX FT-NMR 500 MHz instrument. The compounds were analyzed at 298, 308, 318, and 328 K, and ^{1}H spectra were recorded. All of the proton and carbon spectra were processed using MestReNova software. The CD experiment was carried out at a JASCO 1500 instrument using a 0.2 mm flow cell. The compounds were made in a concentration of 0.1 mg/mL in methanol, and the spectrum was recorded with three accumulations. Analytical and semipreparative HPLC analysis/purification were carried out on a Shimadzu HPLC system having a quaternary pump and a PDA detector using an RP-18e, 125 × 4 mm^2, 5 μm column (Merck, LiChrospher 100) for analytical purposes and a C_{18}, 250 × 10 mm^2, 5 μm column (Dr Maish GmbH, Reprosil Gold 100 XBD) for semipreparative separation. The mobile phase used was water and acetonitrile/methanol with a suitable gradient profile for analysis and purification of synthetic peptides.

HPLC Purity Analysis. The HPLC purity analysis was carried out using the aforementioned analytical column attached to a Shimadzu HPLC system eluted with mobile phase A of 0.1% TFA in water and mobile phase B of acetonitrile. The starting gradient was 10% B, gradually increased to 60% B in 30 min, and stayed at 60% B for 5 min followed by a sharp decrease to 10% B in 2 min and stayed at 10% B for the next 3 min with an overall run time of 40 min. The chromatogram was observed at 214 nm with a flow rate of 1 mL/min and was applied to the entire set of compounds except more nonpolar myristoyl analogue (**41**). Compound **41** was analyzed using a gradient method starting at 30% B, which was gradually increased to 90% B in 20 min and was stayed at 90% B for next 10 min; this was followed by a sharp decrease to 30% B in 3 min and was stayed at 30% B for next 5 min with an overall run time of 38 min, keeping the other conditions the same as stated above.

Cell Culture and Treatment. All of the cell lines used in this study were procured from Sigma-Aldrich, India (ECACC) and grown in RPMI-1640 medium containing 10% FCS and 100 μg/mL kanamycin and streptomycin. All of the cell lines were cultured at 37 °C with a 5% CO_2 gas atmosphere and 95% humidity. Cells grown in the semiconfluent stage (approx. 70% confluent) were treated with test materials dissolved in DMSO, while the untreated control cultures received only the vehicle, i.e., less than 0.2% DMSO.

Cell Proliferation Assay. The MDA-MB-231, T47D, and MCF-7 cell lines at around 70−75% confluence were treated with different concentrations of compound 4 for 48 h. The MTT dye (2.5 mg/mL in PBS) was then added 4 h prior to the termination of the experiment. MTT formazan crystals were dissolved in DMSO (150 μL), and absorbance was measured at a width of 570 nm.[34]

Cell Cycle Analysis. The cell line HL-60 was treated with different concentrations of compound 4 (10, 20, and 30 μM) for 24 h. The cell cycle was analyzed on a flow cytometer using propidium iodide (PI).[34]

Hoechst Staining. Hoechst 33258 staining of cells was done for analyzing the nuclear morphology of cells. A total of 2 × 10^6 cells/3 mL seeded in plates were treated with the given compounds and after the termination of the experiment washed twice with PBS, fixed, and stained with Hoechst 33258 as described earlier.[34] The slides were observed for any nuclear morphological alterations and apoptotic bodies under an inverted fluorescence microscope.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c04038.

Experimental details of compounds; synthesis of compounds **1**−**4**; *N*-truncated analogues; C-terminal truncated analogues; random deletion sequence analogues; N-terminal derivatives; C-terminal derivatives of lipovelutibol D; *L*-isomer of lipovelutibol D; spectroscopic details of compounds **1**−**19** and **21**−**48**; ^{1}H, ^{13}C NMR, HPLC purity results for **1**−**5**, **8**−**10**, **12**−**16**, **18**−**21**, **24**−**25**, **27**−**46**, and **47**−**50**; HRMS data and CD analysis of natural and synthetic compounds **1**−**4**; NMR-VT experiment of NH regions of **1**−**4**; and IC_{50} values of compounds **41**,**42**, and **44** (PDF)

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Notes

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