Missing Selectivity of Targeted 4β-Phorbol Prodrugs Expected to be Potential Chemotherapeutics

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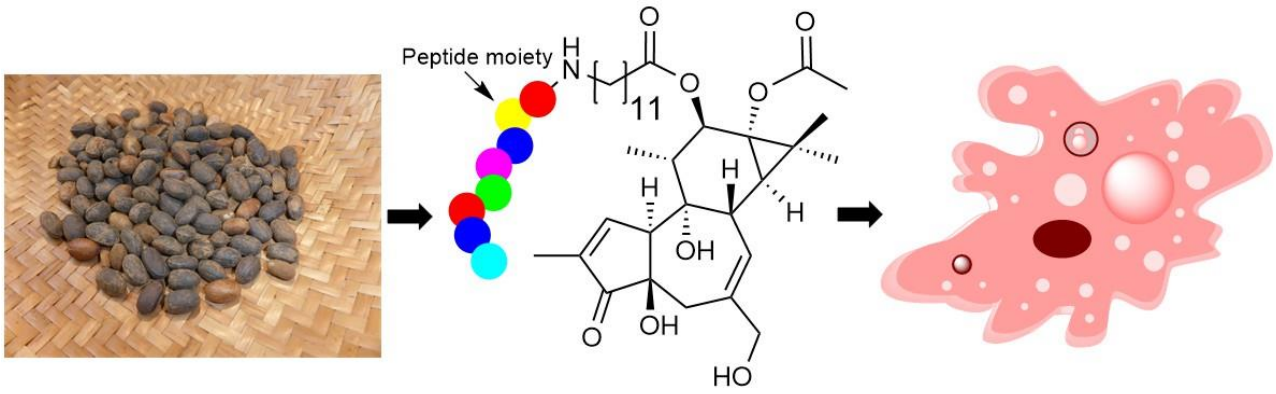


Croton tiglium seeds Phorbol prodrug Selective cancer cell death

Abstract

Targeting cytotoxic 4β-phorbol esters toward cancer tissue was attempted by conjugating a 4β-pborbol derivative with substrates for the proteases prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSMA) expressed in cancer tissue. The hydrophilic peptide moiety was hypothesized to prevent penetration of the prodrugs into cells and prevent interaction with PKC. Cleavage of the peptide in cancer tumors was envisioned to release lipophilic cytotoxins, which subsequently penetrate into cancer cells. The 4β-phorbol esters were prepared from 4β-phorbol isolated from *Croton tiglium* seeds, while the peptides were prepared by solid-phase synthesis. Cellular assays revealed activation of PKC by the prodrugs and efficient killing of both peptidase positive as well as peptidase negative cells. Consequently no selectivity for enzyme expressing cells was found.

Keywords: 4β-Phorbol ester, Protease-assisted targeting, Targeted chemotherapy, Prodrug, Prostate-specific antigen. Prostate-specific membrane antigen,

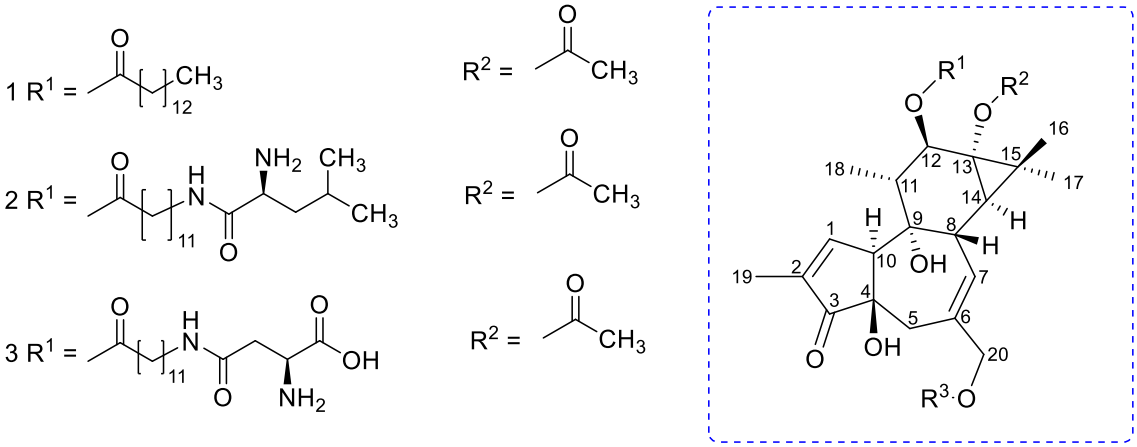
Memoriam: This article is written in memory of the valuable contributions professor Maurizio Botta has offered to medicinal chemistry

Prostate cancer (PCa) is a major cause of death by cancer in men in high-income countries.[1] In the initial stage, PCa mainly consists of cells that are androgen-dependent, and the growth can be retarded by hormone therapy.[2] Unfortunately, in later stages hormone refractory cells dominate (castration-resistant prostate cancer, CRPC).[2, 3] At this stage the use of common chemotherapeutics is complicated by the slow proliferation of the cancer tissue, since chemotherapeutics like taxanes, doxorubicine or vincristine target the proliferative stages of cancer. Thus, selectivity is obtained by the faster division rate for cancer cells.[3-6] Therefore an urgent need for drugs against late-stage PCa exists.

Pre-clinical evidence supports the idea that drugs targeting protein kinase C (PKC) may be useful in treatment of CRPC.[7] The PKC family comprises ten serine/threonine kinases, which can be divided into three groups: i) conventional PKC (cPKCs: -α, -βI, -βII and -γ, ii) novel PKCs (nPKCs: -δ, -ε, -θ and -η) and iii) atypical PKCs (aPKCs: -ζ, -ι and λ) Expression and function of different PKC isoforms are context- and cell type-specific[8-11]. High expression of PKCδ has been reported in prostate cancer, and activation of PKCδ induces apoptosis in LNCaP PCa cells.[9, 12-15] PKCε is generally overexpressed in PCa and downregulation of PKCε induces apoptosis.[16-20]

PKC-activating diterpenoids related to PMA (**1**, Fig. 1) have been in clinical trials.[21-23] Tiglianol tiglate (ECB-46) awaits approval by FDA and EMA for treatment of mast cell tumours in dogs[24] and is entering phase IIA clinical trials for treatment of head and neck squamous cell carcinoma (HNSCC) in humans. Ingenol 3-angelate has under the trade name Picato^R been approved by FDA in 2012 as a topical gel for the treatment of actinic keratosis (preliminary stage of skin cancer).[25, 26] Since PKC is present in virtually all cells, administration of phorbol esters may affect normal physiology in a broad sense. Selectivity of cancer therapies may be obtained by taking advantage of proteases present in tumors.[27] Prostate specific antigen (PSA), a peptidase expressed by the prostate and PCa is a diagnostic marker for prostate cancer and it has been suggested to be involved in cancer invasion and metastasis.[28-32] The missing activity of PSA in the blood caused by complexation with proteins like blood albumin,[3, 27] makes the enzyme a potential facilitator for selective drug delivery. Since both PSA and prostate specific membrane antigen (PSMA) are expressed by PCa even when they become more undifferentiated and anaplastic, they appear to be promising tools in the targeting of toxins for tumors even in CRPC [33, 34]. In the case of thapsigargin (**Tg1**, Supporting Information Fig. S27) selectivity toward cancer tissue was obtained by conjugation of 8-*O*-12-aminododecanoyl-8-*O*-debutanoylthapsigargin with peptides that are substrates for human glandular kallikrein 2, hK2,[35, 36] PSA or PSMA (mipsagargin, **Tg6**, Fig. S27).[37] Mipsagargin has successfully passed clinical trial 2 (For details see Supporting Information paragraph *S3*).[38] Based on the above findings 4β-phorbol esters **4**-**6** (Fig. 1) were designed and expected to display a similar behaviour in the organism as the thapsigargin analogs. By

conjugating the toxin with a substrate for the proteases, penetration into cells is ideally only possible after enzymatic cleavage by either PSA or PSMA.[30, 37] Encouraged by the above-mentioned findings and hypotheses we have attempted to develop prodrugs of 4β-phorbol esters for selective targeting of PSA- and PSMA-expressing cancer cells.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2 R1 =
3 R1 =
R2 =
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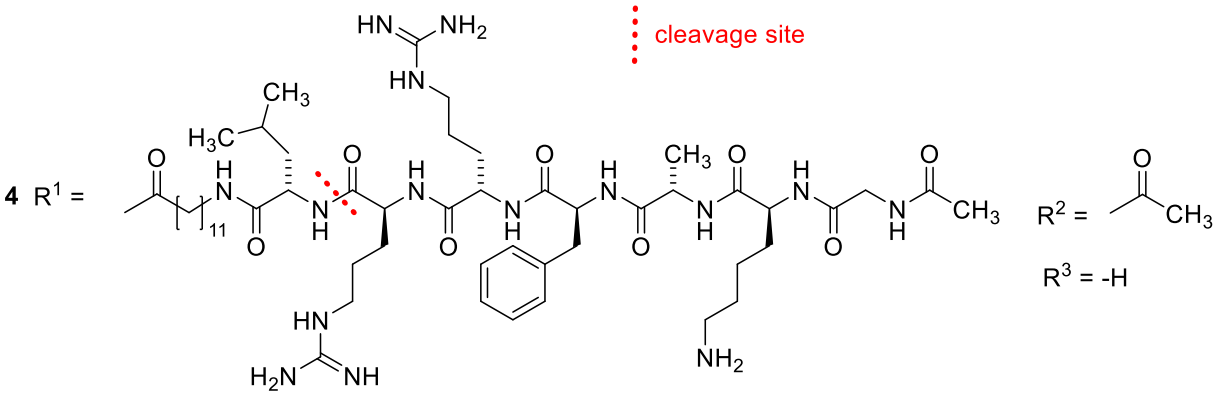
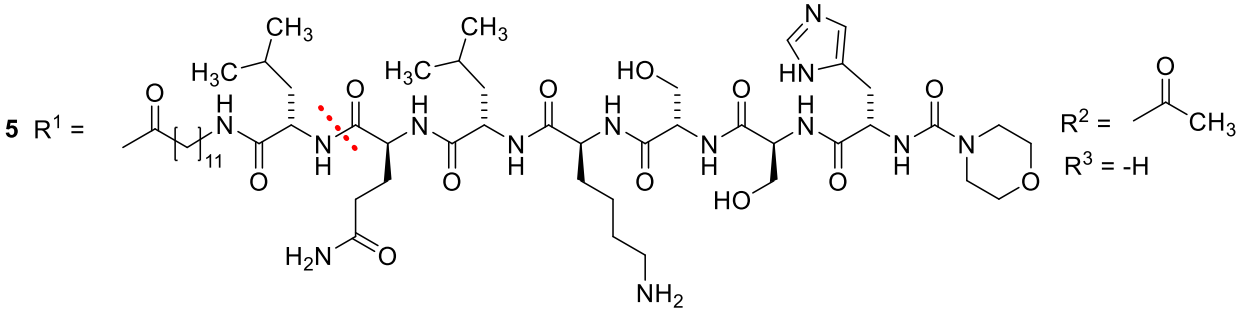
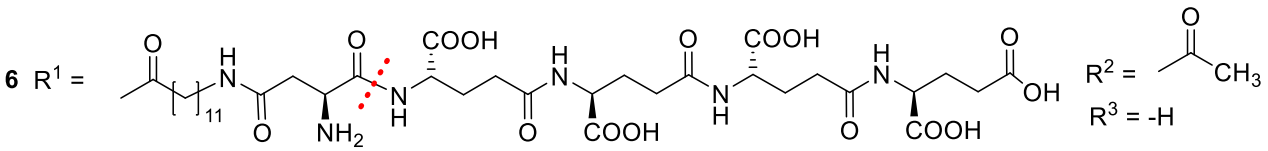
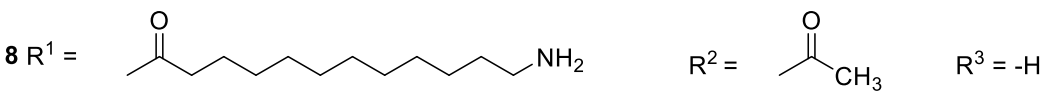

4 R1 =
cleavage site
R2 =
R3 = -H


5 R1 =
R2 =
R3 = -H


6 R1 =
R2 =
R3 = -H

7 $R^1 = R^2 = -H$


8 R1 =
R2 =
R3 = -H

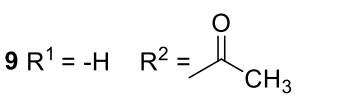

9 R1 = -H
R2 =
R3 = -TBDMS

Fig. 1. Target compounds and starting material: 4β-Phorbol 12-*O*-myristate 13-*O*-acetate (**1**), toxin **2** obtained after cleavage of prodrugs **4** and **5** with hK2 or PSA, respectively, while toxin **3** is obtained after cleavage of prodrug **6** with PSMA. 4β-Phorbol (**7**). Compound **8** is the starting material for synthesis of compounds **2** – **3** and compound **9** for **4** – **6**.

The starting material 4β-phorbol (**7**) was obtained from seeds of *Croton tiglium* L. (Euphorbiaceae) (for details see Supporting Information paragraph S2.5.1). By a few synthetic steps 4β-pborbol was converted into the cytotoxins **2** and **3** via **8** (Supporitn Information S2.5.2). The peptides needed for preparing the prodrugs 4 – **6** were prepared by solid phase syntheses For syntheses and characterization of the 4β-phorbol toxins and prodrugs see Supporting Information S2.5.3 – S2.5.10.

Binding to PKC as Measured by [^{3}H]PDBu Displacement assay. Compounds **2** and **3** as well as prodrugs **4** - **6**) were tested for binding to the C1 domains of PKCα in a 96-well plate filtration assay as described earlier[39] at a concentration range of 0.01-10 μM. All new compounds (i.e., **2** -**6**) displaced [^{3}H]4β-phorbol 12,13-dibutyrate ([^{3}H]PDBu) as efficiently as PMA (**1**) (Fig. 2) except for prodrug **5**, for which an approximately ten times higher concentration was required to achieve a displacement comparable to that of the other compounds. Thus, the presence of a peptide moiety in the prodrugs did not nullify their affinities to the C1 domain of PKCα.

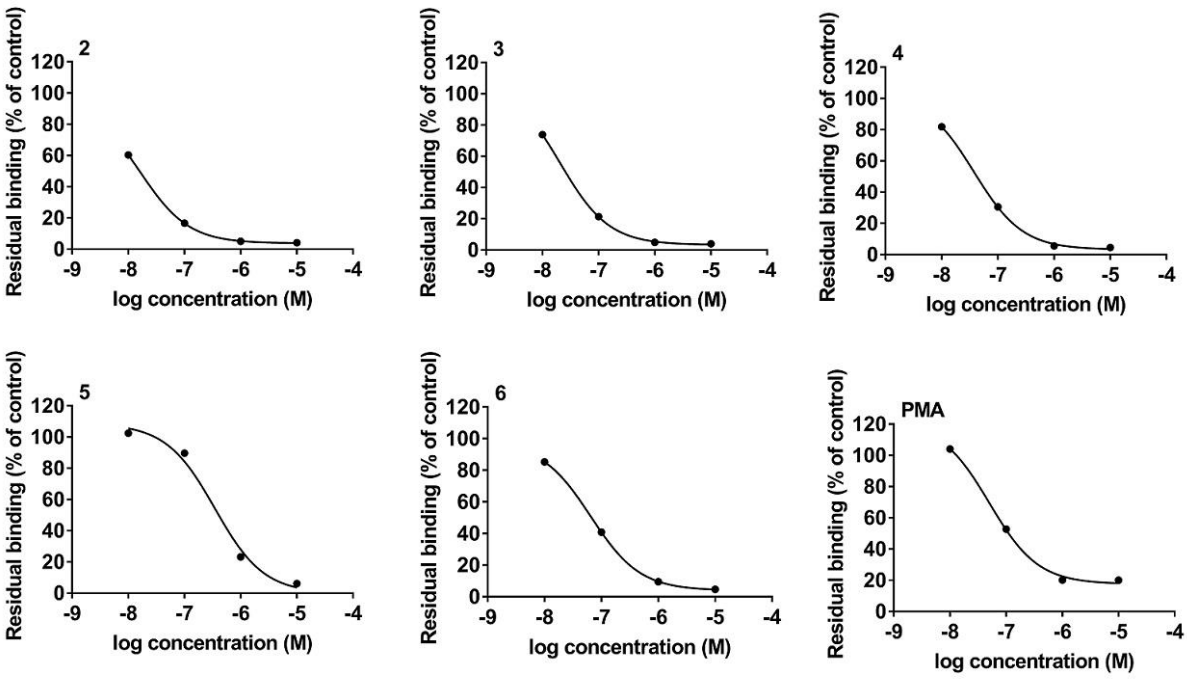


Fig. 2. Displacement binding curves of prodrugs **4** – **6**. Toxins **2** and **3** and PMA. Binding of [^{3}H]PDBu (10 nM) to PKCα was measured in the presence of increasing concentrations of the tested compounds. The PKCα was obtained from a lysate if cells overexpressing the enzyme. The data is presented as mean of residual [3H]PDBu binding (% of control) from three parallel samples in a single representative experiment.

Cell Death as Measured by Cell viability assays. The effect of the compounds on viability of PCa cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Fig.4). The previously established PSMA prodrug mipsagargin (**Tg6)** and the PSMA cleavage product Asp-12-AD-thapsigargin (**Tg3**) were used as reference compounds [37]. The PCa cell lines, used in the present study, represent different types of PCa: androgen-unresponsive DU145 and PC3 cells, which do not express PSA or PSMA, and androgen-responsive 22Rv1 and LNCaP cells, which both are PSA- and PSMA-positive.[40, 41] The maximal effect of the PSA cleavage product **2** with the highest concentration gave rise to a reduction in viability of PSA/PSMA-positive LNCaP and 22Rv1 cells to ~60% and ~10%, respectively, and in PSA/PSMA-negative DU145 and PC3 cells to ~40% and ~30%, respectively (Fig. 3). PSA prodrug **5** reduced the viability to below 50% only in LNCaP cells (~40%) at 20 µM and 40 µM concentrations, whereas PSA prodrug **4** reduced the viability to below 50% at the highest concentration not only in PSA-positive 22Rv1 (to ~25%) but also in PSA-negative PC3 cells (~15%) at the highest concentration. To our surprise, the PSMA cleavage product **3** had almost no effect on cell viability in any of the PCa cell lines. The PSMA prodrug **6**, however, decreased the viability to ~10% at the

highest concentration only in the PSMA-negative PC3 cells (Fig. 3). The reference compound **Tg3** decreased the viability concentration-dependently in all PC cell lines, and its maximal effect (achieved with the highest concentration) was a reduction in viability to ~4% for LNCaP, 27% for 22Rv1, 20% for DU145, and ~15% for PC3 cells. Surprisingly, the other reference compound (i.e., **Tg6**) demonstrated a similar reduction in the viability in PSMA-negative PC3 cells (to ~32%) as seen for the PSMA-positive LNCaP and 22Rv1 cells (to ~35% and ~40%, respectively; Fig. 3).

PMA is known to promote PKC-induced apoptosis in the LNCaP cell line [42]. In accordance with this, the pan-PKC inhibitor Gö6983 was able to dampen the effect of 20 µM of compound **5** in LNCaP cells (Fig. 3), indicating that the cytotoxic effect indeed is PKC-mediated. The compounds did not induce distinct damage to the cell membranes during the 72-h incubation with any of the concentrations as determined by the LDH test (Fig. S2).

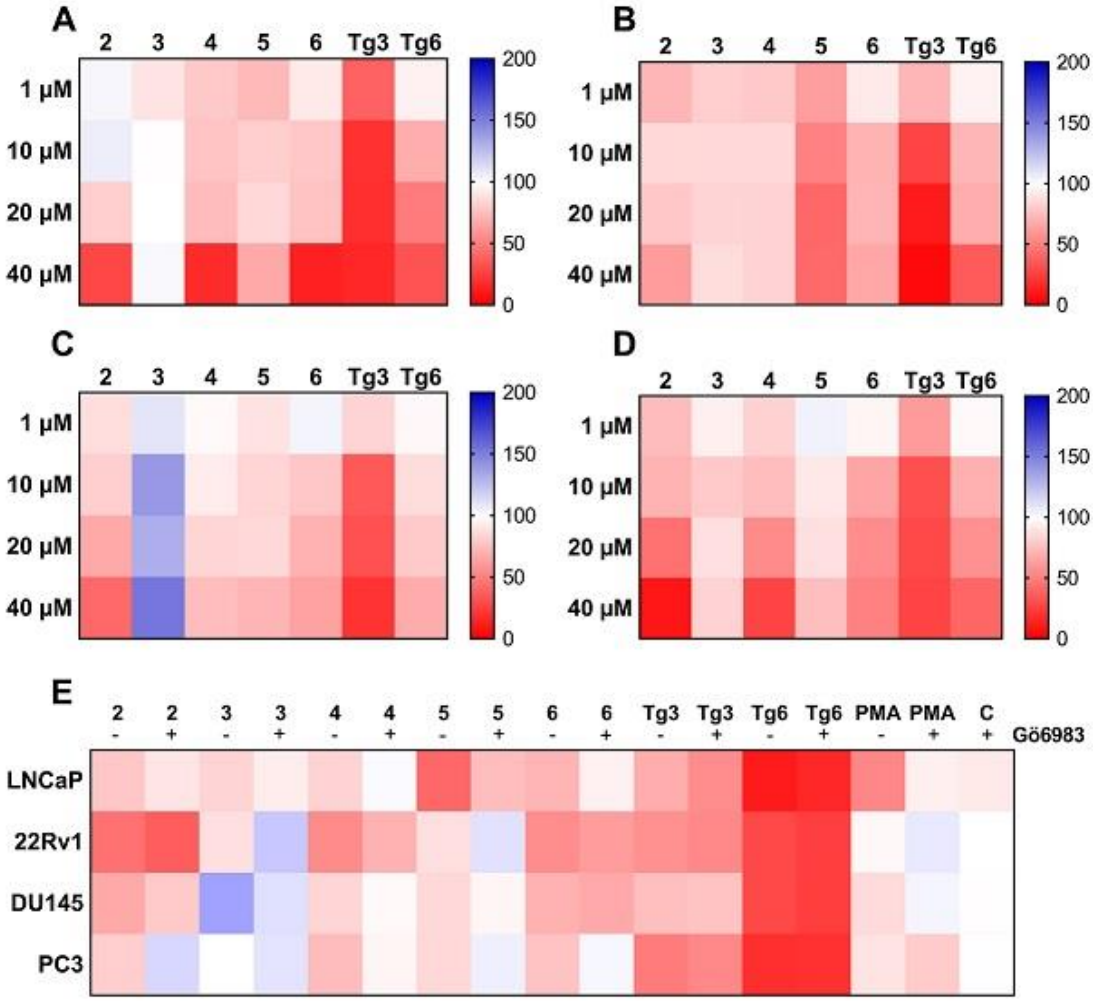


Fig. 3. Effects of phorbol prodrugs **4** - **6**, and cleavage products **2** and **3** as well as **Tg3** and **Tg6** on viability of PCa cell lines. (A) PC3; (B) LNCaP; (C) DU145; (D) 22Rv1 and the effect of PKC inhibitor Gö6983 (1 µM) on the effect of 20 µM of **2** - **6**, **Tg3**, **Tg6** and 100 nM PMA on viability of LNCaP, 22Rv1, DU145 and PC3 cells (E). Cell viability was measured

after 72 h incubation with the compounds by utilizing the MTT assay. The data is presented as mean of cell viability (% of control) (n=3).

Effects on ERK1/2 phosphorylation and protein expression of PKC and PSMA

Extracellular signal-regulated kinases 1 and 2 (ERK1/2) are members of the mitogen-activated protein kinase (MAPK) signalling cascade that controls several cellular processes [43]. PKC-mediated ERK1/2 phosphorylation is one of the initial rapid events in PMA-treated LNCaP cells [42] Since the novel 4β-phorbol-derived compounds compete with PDBu *in vitro*, their ability to modulate ERK activity was investigated in living cells.[44] PSA/PSMA-positive 22Rv1 and PSA/PSMA-negative DU145 PCa cell lines were exposed to 20 µM of compounds **2** - **6**, **Tg3** and **Tg6** and to 10 nM of PMA for 30 min. Phorbol-derived compounds **2** - **5** induced substantial ERK1/2 phosphorylation in 22Rv1 cells (Fig. 4). The ERK1/2 phosphorylation was even more distinct than after PMA exposure, except in cells treated with compound **6**. The phorbol derivatives also induced ERK1/2 phosphorylation in DU145 cells, but the magnitude of the effect was distinctively smaller than in 22Rv1 cells (Fig. 4).

Downregulation of PKC has been suggested to explain the tumor promotion caused by phorbol esters.[10, 45] In addition PMA is able to induce androgen receptor downregulation in PCa cells, which is associated with PSMA downregulation. [46, 47] To understand the effects of our compounds on the expression of PKC and PSMA we decided to investigate the effects of the phorbol derivatives on the expression levels of PKCα and PKCδ and PSMA in 22Rv1 cells. A 24 h exposure to 20 µM **3** and **6** increased the expression of PKCα while the incubation with **2** and **4** had no effect. However, PKCα, PKCδ and PSMA expression was reduced upon 24 h exposure to all compounds, including **3** and **6** (Fig. S4 and Table S3)**.** Indeed, our results support the hypothesis that phorbol ester induced down regulation of PKC is associated with down regulation of PSMA. Probably the PKC activating effects of our toxins caused a down regulation of PKC and PSMA.

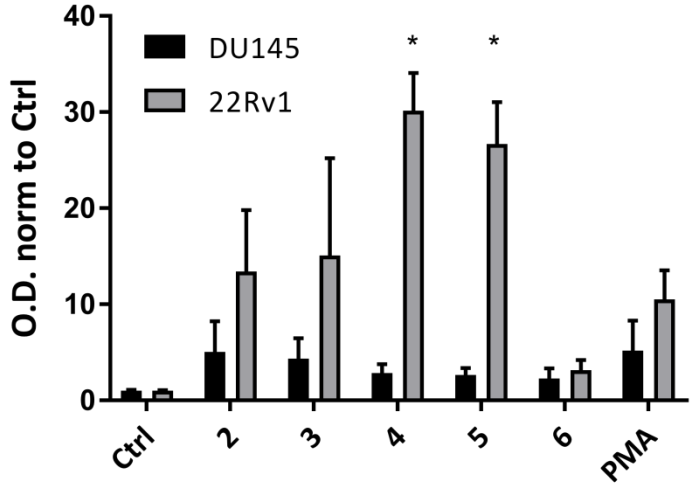


Fig. 4. Effects of phorbol derivatives **2** - **6** on ERK1/2 phosphorylation in PC cells. Quantifications from DU145 and 22Rv1 cells. Data is presented as mean + SEM (N = 3; *P < 0.05 vs ctrl, Welch's t-test). The cells were treated with 20 µM of different phorbol derivatives and PMA for 30 min. The cells were harvested, and then ERK1/2 phosphorylation was analysed by using Western blotting with detection as described in the Experimental Section.

Conclusion

In the present study, we synthesised, characterised and evaluated ability of the PSA/PSMA-activable 4β-phorbol ester prodrugs **4** - **6** and the corresponding cytotoxins **2** and **3** to displace ^{3}H PDBu from PKC and to decrease the viability of PSMA/PSA-positive as well as PSMA/PSA-negative PCa cell lines. In addition, we performed studies on their abilities to increase ERK1/2 phosphorylation. The synthesised PSA/PSMA-activable 4β-phorbol ester prodrugs were designed to contain peptide sequences that are specifically cleaved by either hK2 (i.e., **4**), PSA (i.e., **5**), or PSMA (i.e., **6**). All phorbol-derived compounds showed low nanomolar binding affinity to the C1 domain of recombinant human PKCα, as shown by their displacement of PBDu (Fig. 3). Compound **3** induced cytotoxicity only to a limited extent, which may be explained by the lack of ability to penetrate the cell membrane due to its zwitterionic nature at physiological pH. Analogously, compound **3** and prodrug **6** provoked phosphorylation of ERK1/2 only to a limited extent (Fig. 3 and 4). Disappointingly prodrugs **4** - **6** showed no selectivity for PSA/PSMA-positive cell lines (i.e., LNCaP/22Rv1) over PSA/PSMA-negative cell lines (i.e., DU145 and PC3). This observation strongly infers that despite conjugation to a hydrophilic peptide the prodrugs (compounds **4** - **6**) retain an ability to penetrate cell membranes. The poor activity of **3** and **6** in the viability assay and in the phosphorylation assay indicates that these highly charged molecules can be taken up by the cells only to a limited extent. The results of the present study do not support the hypothesis that the designed PSA/PSMA-targeted prodrugs are capable of providing selective toxicity to PSA/PSMA-expressing PCa cells. Importantly the present results obtained for the known

prodrug mipsagargin (**Tg6**, Supplementary Information Fig. S26) does not support previous observations of selectivity for peptidase expressing cell lines[30] since **Tg6** exhibited clear toxicity both on the PSMA-negative cell line PC3 and on the PSMA-expressing cell lines LNCaP and 22Rv1.[37] A similar poor selectivity has recently been reported by Akinboy et al.[3] fo PSA-targeted *O*-8-(morpholine-4-carbonyl-His-Ser-Ser-Lys-Leu-Phe-Gln-Leu-*N*-12-aminododecanoyl)-*O*-8-debutanoyl-thapsigargin (**Tg5**). The missing selectivity of the 4β-phorbol alanlogs are even more surprising than the missing selectivity of the thapsigargin analogs since the first mentioned are calculated to have lower logP values. In conclusion, the proposed targeted therapy involving conjugation to peptides that are selectively cleaved by proteases present in cancer tissue appears to lack the desired selectivity with 4β-phorbol and thapsigargin analogs.

ASSSOCIATED CONTENT

Supporting information

The Supporting Information is available free of charge on the ACS Publications website at DOI

Experimental procedures, spectral data for all compounds, spectra for al target compounds and supporting biological data are available.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. Ilari Tarvainen and Tomáš Zimmermann has contributed equally to the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

Boc: *tert*-butyloxycarbonyl; CRPC: castration-resistant prostate cancer, 2-CTC: 2-chlorotrityl chloride; 2-Cl-Trt: 2-chlorotrityl; DCM: dichloromethane; DIPEA: *N,N*-diisopropylethylamine; DMAP: 4-(*N*,*N*-dimethylamino)pyridine; DMF: *N,N*-dimethylformamide; EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; Fmoc: fluorenylmethyloxycarbonyl; GPCR: G-protein-coupled receptor; HBTU: 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HFIP: 1,1,1,3,3,3-hexafluoro-2-propanol; $InsP_3$: inositol-1,4,5-trisphosphate; MTT: methylthiazolyldiphenyltetrazolium bromide; MW: microwave; NMP: *N*-methyl-2-pyrrolidone; PCa: prostate cancer; PDBu: phorbol-12,13-dibutyrate; PKC: protein kinase C; PMA: 4β-phorbol 12-myristate 13-acetate; PSA: prostate-specific antigen; PSMA: prostate-specific membrane antigen; PyBOP: benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate; SPPS: solid-phase peptide synthesis; TBDMS: *tert*-butyldimethylsilyl; THF: tetrahydrofuran

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Supporting Information for

Missing Selectivity of Targeted 4β-Phorbol Prodrugs Expected to be Potential Chemotherapeutics

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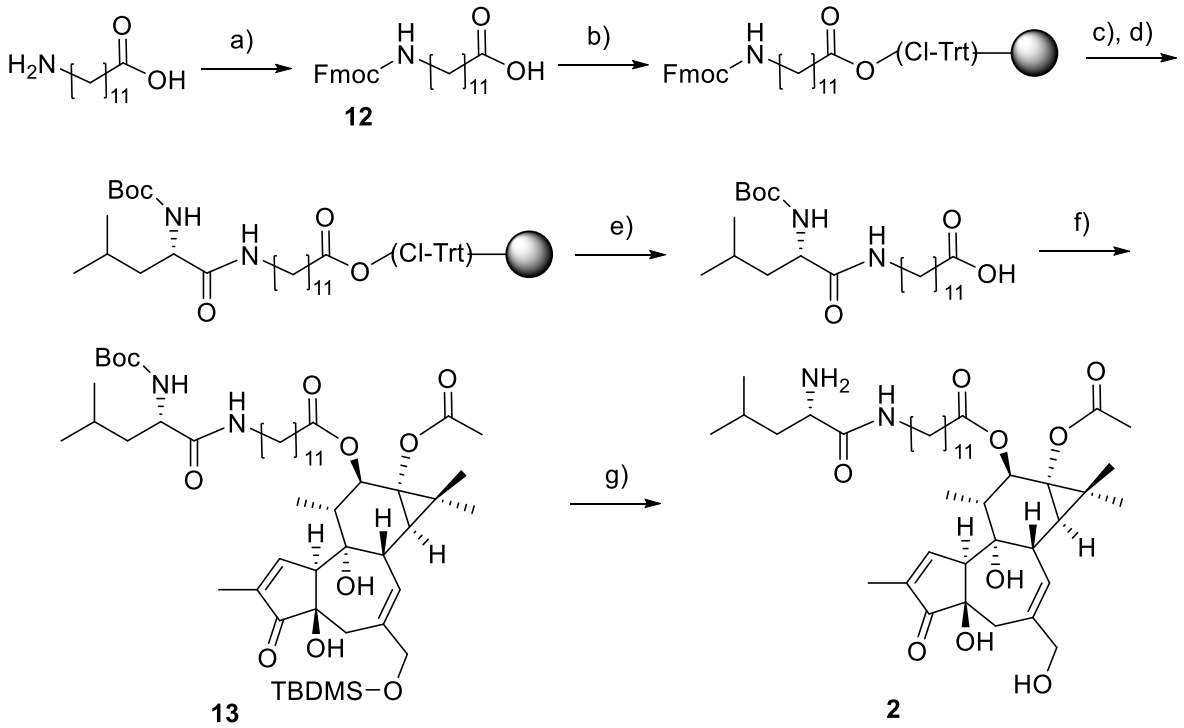
Contents:

S1: Syntheses of Compounds 2 - 6

S1.1: Synthesis of Cytotoxin 2

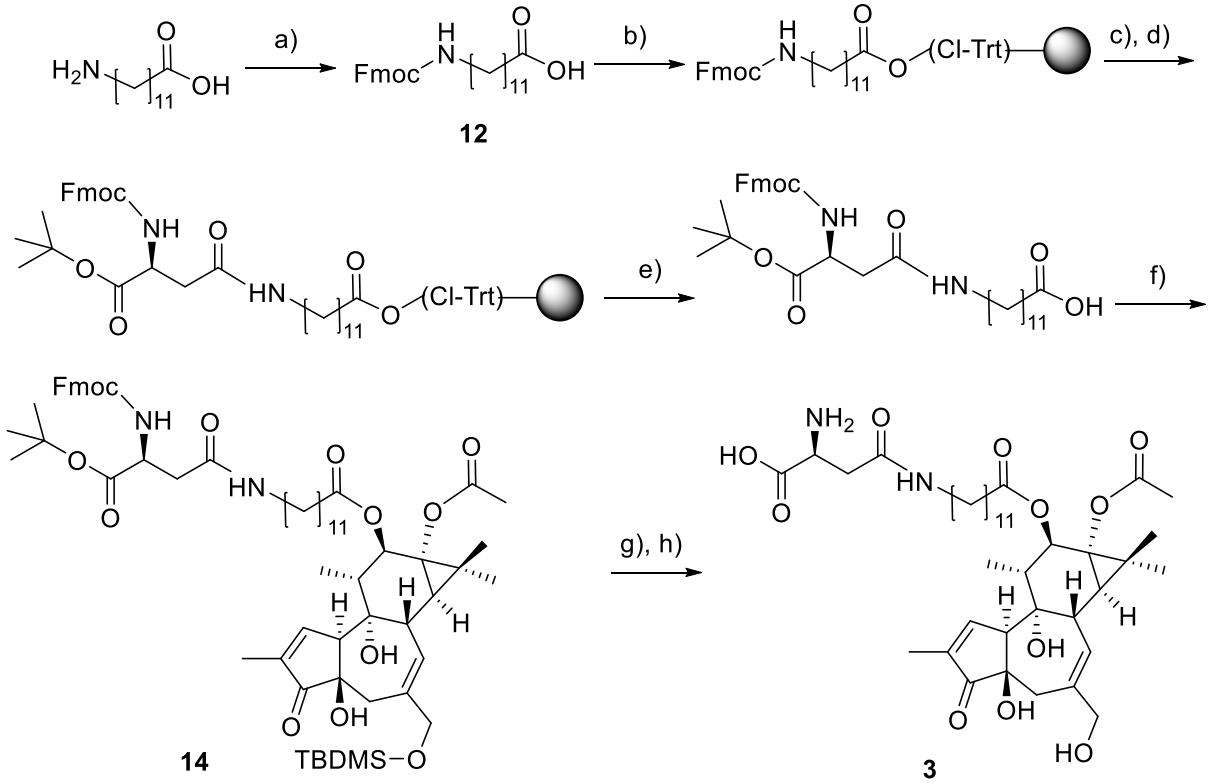
By using solid-phase synthesis for the conjugation several protection-deprotection steps were avoided. First 12-Aminododecanoic acid was loaded onto a 2-chlorotrityl chloride (2-CTC) resin, and upon removal of the fluorenylmethyloxycarbonyl (Fmoc) group Boc-Leu-OH was attached. The resulting resin-bound Boc-Leu-12-aminododecanoic acid was released from the resin under mildly acidic conditions, and was then coupled to **9** by Steglich esterification using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 4-(*N*,*N*-dimethylamino)pyridine (DMAP) to give compound **2** (Scheme 1).



Scheme S1. Synthesis of compound **2.** Reagents and conditions: (**a**) Fmoc-OSu, $NaHCO_3$, H_2O–acetone (1:1), 23 °C, 20 h (43%); (**b**) 2-CTC resin, DIPEA, 23 °C, 3 h; (**c**) 20% Piperidine in DMF, 23 °C, 2×20 min; (**d**) Boc-Leu-OH, PyBOP, DIPEA, DMF, 4 h, 23 °C; (**e**) 20% HFIP in DCM, 23 °C, 3×30 min; (**f**) EDC, DMAP, compound **9**, 23 °C (86%), 15 h, DCM–THF (1:1); (**g**) 23 °C, 3 h, TFA–DCM (1:1) (30%).

S1.2: Synthesis of Intermediate 3

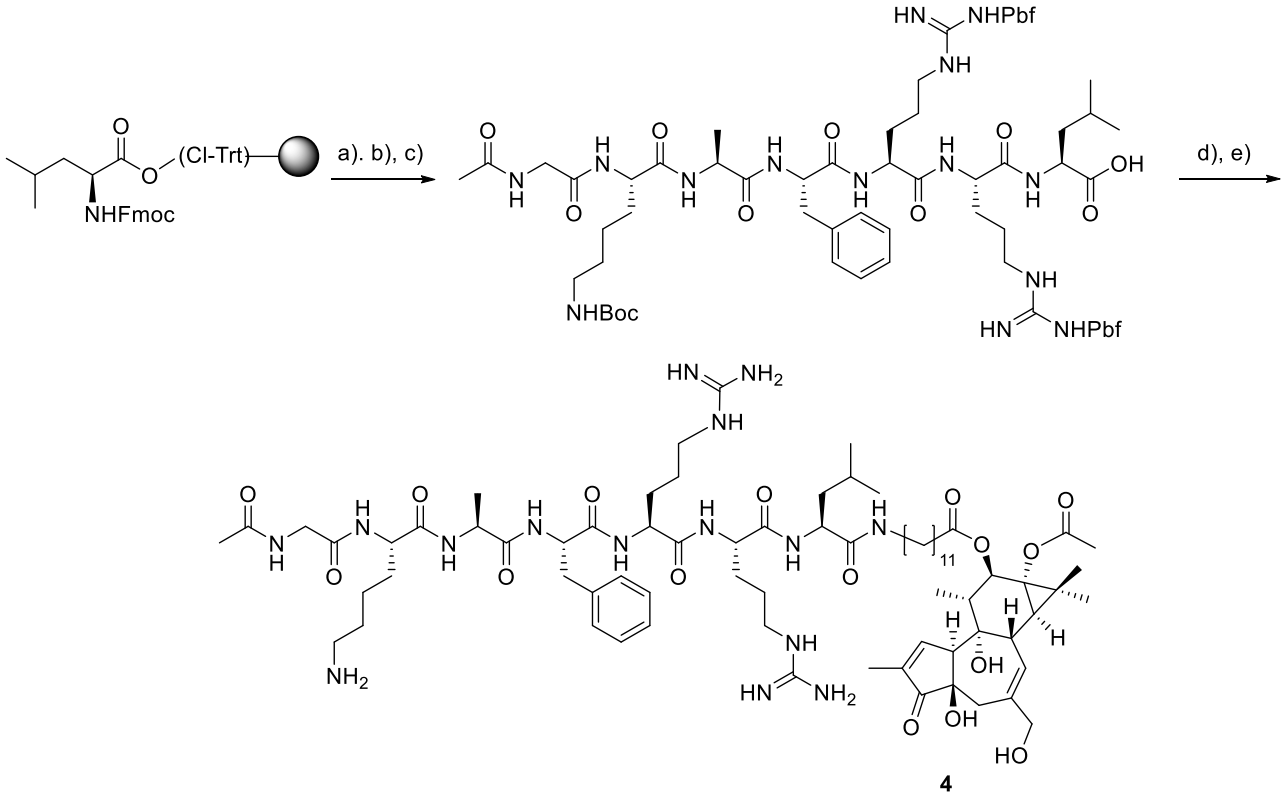
Fmoc-Asp-α-O*t*Bu was coupled to resin-bound 12-aminododecanoic acid, and the resulting Fmoc-β-Asp(O*t*Bu)-*N*-12-aminododecanoic acid was released from the resin. In order to avoid undesired DMAP-catalyzed removal of the Fmoc protecting group during synthesis of **14** Fmoc-β-Asp(O*t*Bu)-12-aminododecanoic acid was esterified with **9** by using a minimum amount of DMAP. The Boc and TBDMS groups were removed simultaneously with TFA, while the Fmoc group was deprotected with diethylamine in DCM to provide target compound **3** (Scheme 2).



Scheme S2. Synthesis of compound **3.** Reagents and conditions: (**a**) Fmoc-OSu, $NaHCO_3$, H_2O–Acetone (1:1), 23 °C, 20 h (43%), 12-Aminododecanoic acid; (**b**) 2-CTC resin, DIPEA, 23 °C, 3 h; (**c**) 20% Piperidine in DMF, 23 °C, 2×20 min; (**d**) Fmoc-Asp-O*t*Bu, PyBOP, DIPEA, DMF, 4 h, 23 °C; (**e**) 20% HFIP in DCM, 23 °C, 3×30 min; (**f**) EDC, 10 mol% DMAP, compound **9**, 23 °C, 25 h, DCM–THF (1:1) (42%); (**g**) 23 °C, 1 h, TFA–DCM (1:1); (**h**) 23 °C, 1 h, 20% Et_2NH in DCM (62%).

S1.3: Synthesis of prodrug 4

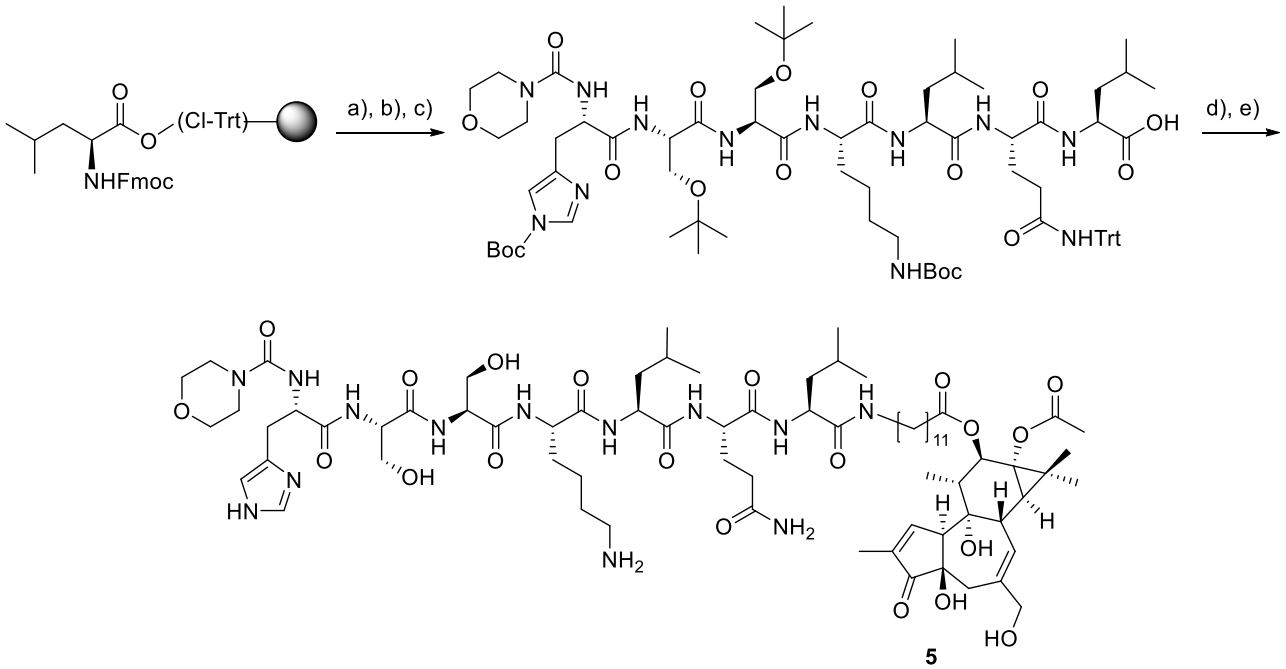
The peptide sequences Gly-Lys-Ala-Phe-Arg-Arg-Leu and His-Ser-Ser-Lys-Leu-Gln-Leu were assembled on an Fmoc-Leu-preloaded 2-chlorotrityl resin by using a CEM Liberty Blue™ automated microwave (MW) peptide synthesizer. The protected resin-bound peptide Gly-Lys-Ala-Phe-Arg-Arg-Leu was end-capped by acetylation. After washing the capped and protected peptide was released from the resin, and then the peptide was coupled in solution to **8**. Finally, the protecting groups were removed with TFA to give prodrug **4** (Scheme 3).



Scheme S3. Synthesis of phorbol prodrug **4** (substrate for hK2). (**a**) SPPS of protected Gly-Lys-Ala-Phe-Arg-Arg-Leu on a MW peptide synthesizer; (**b**) NMP–DIPEA–Ac_2O (3:2:1), 23 °C, 2×10 min (**c**) 20% HFIP in DCM, 23 °C, 3×30 min; (**d**) 12-*O*-(12-Aminododecanoyl)-13-*O*-acetyl-4β-phorbol (**8**), PyBOP, DIPEA, 40 °C, 26 h; (**e**) TFA–DCM (2:1), H_2O (few drops), 23 °C, 80 min.

S1.4: Synthesis of Prodrug 5

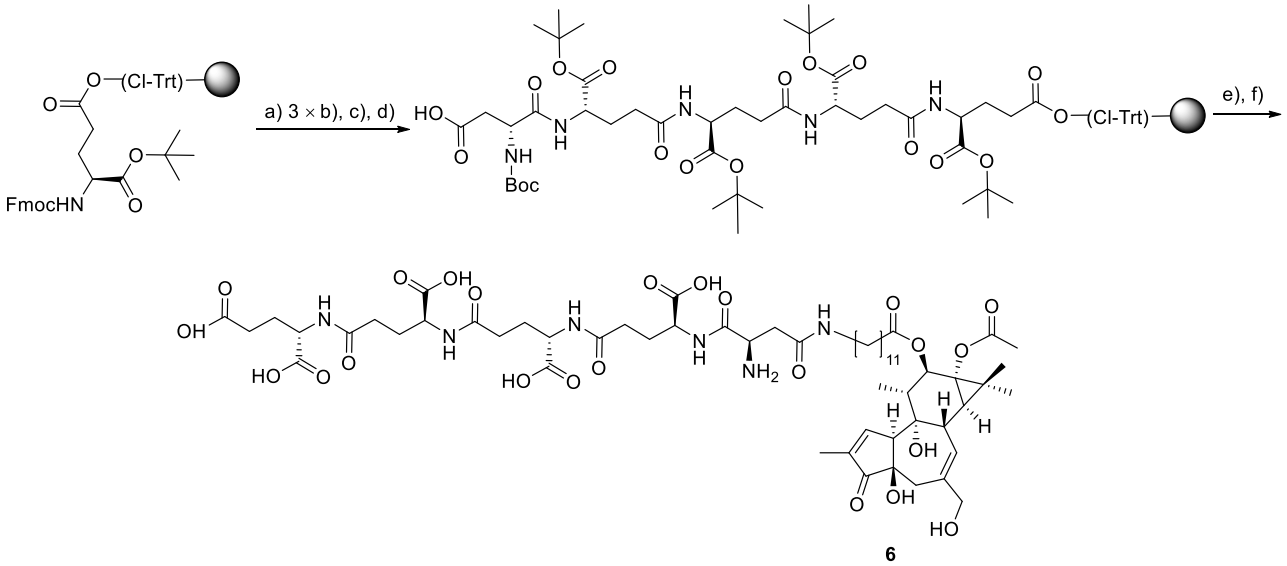
Upon MW-assisted assembly the protected resin-bound peptide His-Ser-Ser-Lys-Leu-Gln-Leu was modified with a urea moiety by reaction with morpholine-4-carbonyl chloride to give the protected peptide of prodrug **5**. After release from the resin the end-capped protected peptide was coupled in solution with **8**. Acid-labile protecting groups were removed with TFA to give prodrug **5** (Scheme 4).



Scheme S4. Synthesis of phorbol prodrug **5** (substrate for PSA). (**a**) SPPS of protected His-Ser-Ser-Lys-Leu-Gln-Leu on a MW peptide synthesizer; (**b**) Morpholine-4-carbonyl chloride–Et_3N–NMP (1:4:15), 40 °C, 3 h; (**c**) 20% HFIP in DCM, 23 °C, 3×30 min; (**d**) 12-*O*-(12-aminododecanoyl)-13-*O*-acetyl-4β-phorbol (**8**), PyBOP, DIPEA, 23 °C, 21 h; (**e**) TFA–DCM (1:1), H_2O (few drops), 23 °C, 1 h (13%).

S1.5: Synthesis of prodrug 6

Synthesis of protected γ-Glu-γ-Glu-γ-Glu-γ-Glu-α-Asp is depicted in Scheme 5. Since sterical hindrance was expected to be an issue in the on-resin coupling with **8**, a downloading of the CTC resin was performed, and the synthesis was carried out manually. Fmoc-Glu-O*t*Bu was loaded onto the CTC resin, and the resulting loading was estimated before continuing the SPPS. The preloaded resin was placed into a syringe fitted with a polypropylene filter, and three additional γ-Glu residues were attached, followed by coupling of Boc-Asp(All)-OH. The orthogonal protection scheme enabled selective removal of the allyl protecting group. Gratifying, the on-resin coupling of **8** to the peptide proceeded satisfactorily. After side-chain deprotection and cleavage from the resin with TFA **6** was obtained (Scheme 5).



Scheme S5. Synthesis of phorbol prodrug **6** (substrate for PSMA). Reagents and conditions: (**a**) 20% Piperidine in DMF, (**b**) Fmoc-Glu-O*t*Bu, PyBOP, DIPEA, DMF, 23 °C, 16 h, followed by 20% piperidine in DMSO; (**c**) Boc-Asp(All)-OH, PyBOP, DIPEA, DMF, 23 °C, 18 h; (**d**) $Me_2N{\cdot}BH_3$, $Pd(PPh_3)_4$ in DCM, 23 °C, 6 h; (**e**) 12-*O*-(12-Aminododecanoyl)-13-*O*-acetyl-4β-phorbol (**8**), PyBOP, DIPEA, DCM, 23 °C, 18 h; (**f**) TFA–DCM (1:1), H_2O (few drops), 1 h (21%).

S2: Experimental section

S2.1. Starting material, reagents, cells and solvents

4β-Phorbol (**7**) was obtained from seeds of *Croton tiglium* purchased from Herbalveda UK, East Harrow Middlesex by extraction and solvolysis [1] while *O*-8-(Leu-*N*-12-aminododecanoyl-)-8-*O*-debutanoyl-thapsigargin (**Tg2**), *O*-8-(β-Asp-*N*-12-aminododecanoyl)-8-*O*-debutanoyl-thapsigargin (**Tg3**), *O*-8-(Boc-*N*-12-aminododecanoyl)-8-*O*-debutanoyl-thapsigargin (**Tg1**) were available in our lab. Mipsagargin (**Tg6**) was prepared as already reported [1] All Fmoc-protected standard amino acids, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), *N,N*-diisopropylethylamine (DIPEA), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) and 2-chlorotrityl chloride (CTC) resin (loading: 1.0–1.6 mmol/g) were purchased from Iris Biotech GmbH (Marktredwitz, Germany). Borane dimethylamino complex, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phorbol 12-myristate 13-acetate (PMA) phosphatidyl-L-serine (PS; product number: P6641), bovine immunoglobulin G (IgG), the pan-PKC inhibitor Gö6983 and $Pd(PPh_3)_4$ were purchased from Sigma-Aldrich. Boc-Asp(All)-OH and Fmoc-Glu-O*t*Bu were purchased from Bachem AG (Bubendorf, Switzerland). All solvents and deprotection and cleavage reagents were of synthesis grade purchased from Iris Biotech GmbH (Marktredwitz, Germany). Solvents for column chromatography, HPLC, HRMS and HR-MALDI-TOF were of HPLC grade purchased from VWR International. [20-^{3}H]Phorbol-12,13-dibutyrate ([^{3}H]PDBu) (20 Ci/mmol) was acquired from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). LNCaP22Rv1, DU145, and PC3 cells were from ATCC (Manassas, VA, USA) (HTB-81; CRL-2505; CRL-1740 and CRL-1435). Protease inhibitors (Complete Protease Inhibitor Cocktail Tablets) were from Roche (Mannheim, Germany), and the Optiphase SuperMix liquid scintillant was from PerkinElmer (Groningen, The Netherlands). *Croton tiglium* seeds were purchased from Herbalveda UK, East Harrow Middlesex, UK.

S2.2. Solid-phase peptide synthesis (SPPS)

Protected peptide **P3** (for numbering see Supplementary data) was synthesized by manual SPPS by using a 2-Cl-Trt resin preloaded with Fmoc-Glu-O*t*Bu. Peptides **P1** and **P2** were assembled on a 2-CTC resin preloaded with Fmoc-Leu-OH by using a Liberty Blue™ automated microwave peptide synthesizer (CEM Corp., Matthews, NC, USA) following an Fmoc/tBu protocol. In all cases, a 2-CTC resin (loading: 1.0–1.6 mmol/g) was used as the solid phase. Couplings were performed by using 0.2 M solutions of N^{α}-Fmoc-protected amino acid building blocks (5 equiv; with acid-labile *t*Bu/Trt/Boc/Pbf as side-chain protecting groups) in DMF in combination with a 0.5 M solution of HBTU (5.0 equiv) as a coupling reagent and a 2 M solution of DIPEA in NMP as the activator base.

Fmoc deprotection was performed with a 20% solution of piperidine in DMF. Peptide Gly-Lys-Ala-Phe-Arg-Arg-Leu was synthesized by using double couplings (each for 15 min) at 45 °C and triple couplings of Arg. Peptide His-Ser-Ser-Lys-Leu-Gln-Leu was synthesized by using single couplings for 15 min at 45 °C. Fmoc removal was performed by repeated treatment with a 20% solution of piperidine in DMF at 45 °C for 30 s and 180 s. Protected products were cleaved from the resin with a 20% solution of HFIP in DCM. Subsequently the protecting groups were removed with TFA added a few drops of H_2O in DCM.

S2.3. Compound purification and characterization

S2.3.1. *HPLC*

Water used for analytical and preparative HPLC was filtered through a 0.22 µm Millipore membrane filter. All final products were purified by reversed phase preparative HPLC on a Phenomenex Luna C18(2) column (250 mm × 21.2 mm; 5 µm particle size) on a Shimadzu Prominence system by using an aqueous MeCN gradient with 0.1% TFA added (eluent A: 5:95 MeCN–H_2O + 0.1% TFA, eluent B: 95:5 MeCN–H_2O + 0.1% TFA). Elution was performed with linear gradients during 20 min at a flow rate of 20 mL/min with UV detection at λ = 220 nm. Purity was determined by analytical HPLC on a Phenomenex Luna C18(2) HTS column (100 mm × 3.0 mm; 2.5 µm particle size) using a Shimadzu Prominence and Shimadzu Nexera system with the same eluents as used for preparative HPLC and a flow rate of 0.5 mL/min. All tested compounds had a purity of at least 95%.

S2.3.2. NMR spectroscopy

^{1}H and ^{13}C NMR spectra were recorded on a 600 MHz Bruker Avance III HD spectrometer equipped with a cryogenically cooled 5 mm dual probe or on a 400 MHz Bruker Ascend spectrometer. Samples were dissolved in methanol-d_4 (Cambridge Isotope Laboratories, Tewksbury, USA) and analyzed at 300 K. The residual solvent peak was used as internal reference (methanol-d_4: δ_C = 49.00; δ_H = 3.31). Coupling constants (*J* values) are given in hertz (Hz). Multiplicities are reported as follows: singlet (s), doublet (d), triplet (t), quartet (q), quintet (quin) and multiplet (m). NMR signals are assigned according to the standard numbering of the phorbol skeleton as displayed in Fig. 1 for PMA (**1**).

S2.3.3. Mass spectrometry

High-resolution mass spectra were recorded on a Bruker MicroTOF-Q LC mass spectrometer equipped with an electrospray ionization source or on a Quadropole MS detector or via MALDI-TOF on a Bruker SolariX XR in MALDI mode. The analyses were performed in positive ionization mode to give peaks of $[M + nH]^{n+}$.

S2.4. Biological assays

S2.4.1. Cell culture

LNCaP, 22Rv1 and DU145 were cultured in RPMI1640 medium (Cat #1060120, MP Biomedicals, Santa Ana, CA, USA), PC3 cells in Ham's F12K (Kaighn's modification, Cat #21127022; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) medium which were supplemented with 10% foetal bovine serum, 100 $U \cdot mL^{-1}$ penicillin, and 100 $\mu g \cdot mL^{-1}$ streptomycin (all from Gibco). Cell cultures were maintained in a humidified incubator with 5% CO_2 at 37 °C. All experiments were done in the above-described cell culture media.

S2.4.2. Displacement assay

Recombinant human PKCα protein was produced in baculovirus-infected Sf9 cells as described previously [2]. The cells were harvested two days after infection, washed with phosphate-buffered saline (PBS) (pH 7.4), and the resultant cell pellets were frozen. Subsequently the Sf9 cells were suspended in buffer containing 25 mM Tris-HCl (pH 7.5), 0.5 mM EGTA, 0.1% Triton X-100, and protease inhibitors to prepare a crude cell lysate. Following a 30-min incubation on ice, the lysate was centrifuged at 16,000 *g* for 15 min at 4 °C and the supernatant representing the soluble fraction was collected. The protein content of the supernatant was determined with a Bradford assay.[3] The ability of the compounds to compete in binding to the regulatory domain of PKCα with tritium-labelled phorbol ester [^{3}H]PDBu was determined according to [4]. Protein (20 µg/well) from the supernatant was incubated with different concentrations of the test compounds and 25 nM of [^{3}H]PDBu for 10 min at room temperature in a 96-well Durapore filter plate (Millipore, cat. no. MSHVN4B50, Carrigtwohill, Ireland) in a total volume of 125 µL. The final concentrations in the assay were: 20 mM Tris-HCl (pH 7.5), 40 µM $CaCl_2$, 10 mM $MgCl_2$, 400 µg/mL bovine IgG, 25 nM [^{3}H]PDBu, and 0.1 mg/mL phosphatidyl-L-serine (1,2-diacyl-*sn*-glycero-3-phospho-L-serine). Proteins were then precipitated by the addition of 125 µL of cold 20% poly(ethylene glycol) 6000. After 15 min of incubation on a plate shaker at room temperature the filters were washed six times using a vacuum manifold with buffer containing 20 mM Tris-HCl (pH 7.5), 100 µM $CaCl_2$ and 5mM $MgCl_2$. The plates were dried and 25 µL of Optiphase SuperMix liquid scintillant was added to each well. Radioactivity was measured using Wallac Microbeta Trilux microplate liquid scintillation counter (PerkinElmer, Waltham, MA, USA) after an equilibration period of three hours. All tested compounds were dissolved in DMSO and diluted with the buffer to give the same final DMSO concentration in the binding assay (4%) in each well. PMA (1 µM) was used as a positive control to obtain maximum displacement in all assays. Since nonspecific binding was always ≈5%, only the total binding was measured. The results were calculated as a percentage of DMSO control from the same plate.

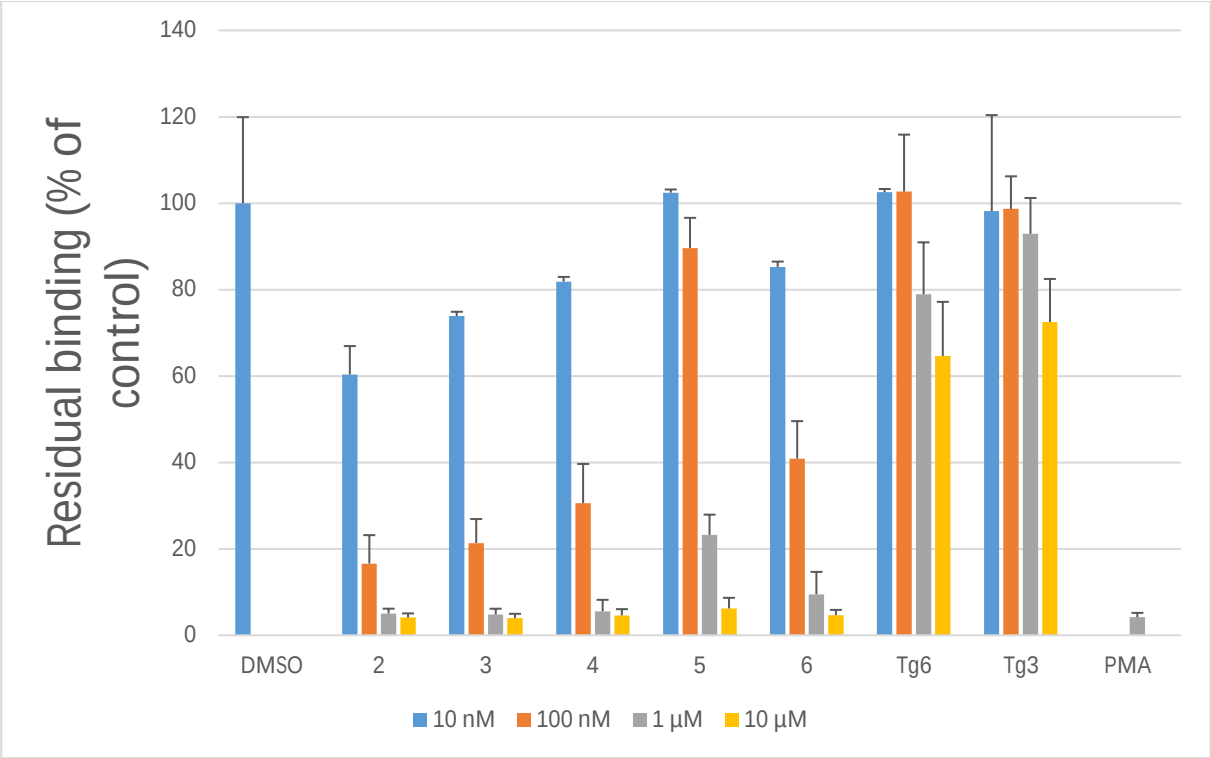


Fig. S1 Displacement assay data presented as mean +SEM (N=3) of residual [^{3}H]PDBu binding (% of control).

Table S1 Displacement assay numerical data presented as averages from three individual experiments and three parallel wells in each experiment (% of control).

		Ctrl	2	3	4	5	6	Tg3	Tg6	PMA
AVG	10 nM	100.00	60.38	73.87	81.85	102.44	85.23	102.55	98.18	
	100 nM		16.60	21.35	30.57	89.61	40.90	102.70	98.68	
	1 µM		5.04	4.80	5.57	23.24	9.49	78.94	92.92	4.17
	10 µM		4.11	3.97	4.59	6.19	4.65	64.65	72.47	
SEM	10 nM	19.91	6.56	1.01	1.10	0.74	1.25	0.74	22.21	
	100 nM		6.57	5.55	9.09	7.01	8.66	13.17	7.54	
	1 µM		1.11	1.34	2.64	4.68	5.18	12.00	8.30	1.02
	10 µM		0.96	1.00	1.45	2.47	1.23	12.52	10.00	

S2.4.3. Cell viability assays

Cell viability was determined using mitochondrial oxidoreductase activity assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)]. Lactate dehydrogenase (LDH) test was used to measure the amount of LDH released from cells with compromised cell membrane integrity. The PSA/PSMA-positive LNCaP and 22Rv1 and PSA/PSMA-negative DU145 and PC3 human PC cells were plated on 96-well plates at 6000–8000 cells per well in serum-supplemented media and exposed to the compounds for 72 h, after which the LDH assay was carried out using 50 µL samples of cell culture media and the MTT assay with the cells. For the LDH assay, 50 µL of LDH substrate solution (1.3 mM β-nicotinamide adenine dinucleotide, 660 µM iodonitrotetrazolium, 54 mM L(+)-lactic acid, 280 µM phenazine methosulfate (all from Sigma-Aldrich) in 0.2 M Tris/HCl, pH 8.2) was added to the media samples. After a 30-min incubation at room temperature the reaction was stopped by adding 50 µL of a 1 M solution of acetic acid in water. The absorbance was measured at 490 nm. Background absorbance was measured from the wells without cells. Untreated cells were used as controls for spontaneous LDH release, and maximal LDH release was determined from cells lysed with 0.9% Triton X-100. In the MTT assay, solution was added to the cells at 0.5 mg/mL. The cells were incubated in cell culture conditions for 2 h, after which cell culture media was aspirated and replaced with 200 µL DMSO. The absorbance was then measured at 550 nm with absorbance at 650 nm subtracted as background. Some of the cells were first pre-incubated with the PKC inhibitor Gö6983 for 10 min and then exposed to compounds (20 µM) for 72 h.

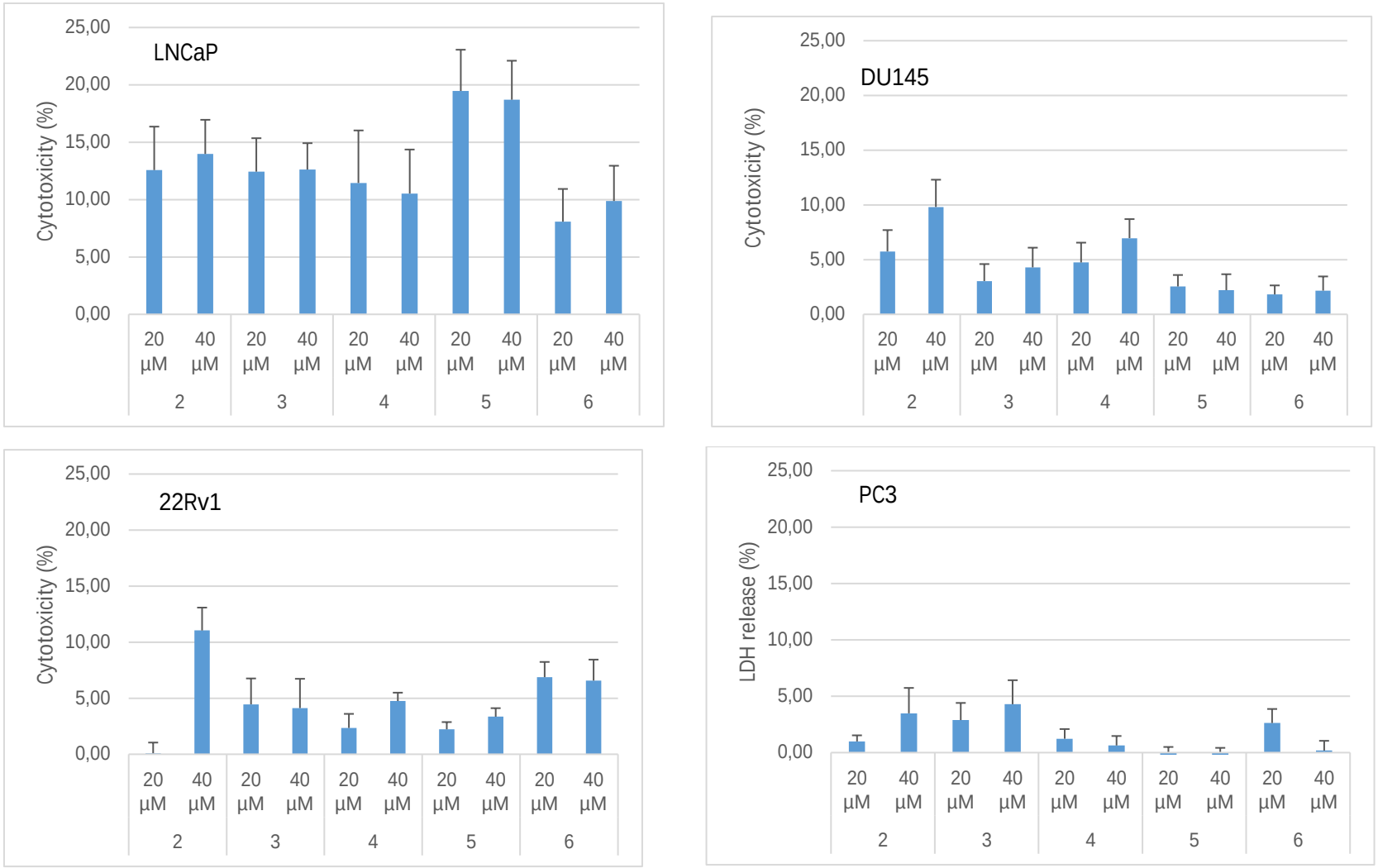


Fig. S2 Necrotic cell death in different cell types after 72 h exposure to the test compounds, measured by LDH assay. Results are presented as mean + SEM (n = 3).

Table S2 Numerical LDH data from three individual experiments and three parallel wells in each experiment.

LNCaP	2		3		4		5		6	
	20 µM	40 µM	20 µM	40 µM	20 µM	40 µM	20 µM	40 µM	20 µM	40 µM
	9.05	17.30	11.34	13.37	18.91	17.02	18.13	18.36	16.01	16.89
	8.99	10.68	20.43	18.75	17.93	15.88	18.28	17.43	13.80	16.66
	8.59	10.21	15.76	15.21	17.62	13.71	18.51	18.23	12.46	17.20
	6.58	8.09	12.35	7.82	15.14	13.71	25.52	26.51	2.82	5.04
	7.63	9.29	7.59	9.66	15.52	14.57	29.01	25.35	3.54	6.24
	8.58	10.38	7.14	10.88	14.78	14.46	26.56	25.54	3.28	6.04
	21.86	19.97	-1.03	-0.48	1.29	1.49	12.80	12.13	8.14	6.79
	21.49	19.70	0.73	1.42	0.19	2.76	14.78	12.99	7.45	7.52
	20.40	20.19	-0.22	1.09	1.64	1.13	11.63	11.79	5.21	6.53
AVG	12.57	13.98	12.43	12.62	11.45	10.53	19.47	18.70	8.08	9.88
STDEV	6.56	5.16	5.05	3.99	7.93	6.64	6.22	5.89	4.93	5.32
SEM	3.79	2.98	2.92	2.30	4.58	3.84	3.59	3.40	2.85	3.07

22Rv1	2		3		4		5		6	
	20 µM	40 µM	20 µM	40 µM	20 µM	40 µM	20 µM	40 µM	20 µM	40 µM
	-1.54	7.57	1.09	0.68	-0.55	3.79	1.25	1.04	4.32	3.40
	0.10	9.47	3.06	1.89	-1.07	3.03	1.42	1.98	4.62	3.42
	-1.21	10.75	2.75	3.02	0.54	4.85	1.53	2.12	4.92	4.61
	-1.54	7.17	1.12	0.53	3.18	5.21	3.68	4.22	6.14	6.17
	-1.34	11.26	1.55	1.72	2.50	3.90	3.09	4.12	5.82	4.44
	-0.25	10.48	2.45	1.94	3.18	3.43	4.19	4.94	6.55	4.89
	1.75	11.57	11.93	13.68	4.45	6.79	1.66	3.59	10.62	10.35
	2.32	11.76	9.83	9.68	4.41	5.49	1.29	3.69	9.55	11.43
	2.44	19.37	6.33	3.93	4.47	6.23	1.90	4.45	9.35	10.44
AVG	0.08	11.04	4.45	4.12	2.35	4.75	2.22	3.35	6.88	6.57
STDEV	1.67	3.54	4.00	4.53	2.18	1.30	1.12	1.32	2.36	3.25
SEM	0.97	2.04	2.31	2.61	1.26	0.75	0.65	0.76	1.36	1.87

DU145	2		3		4		5		6	
	20 μM	40 μM	20 μM	40 μM	20 μM	40 μM	20 μM	40 μM	20 μM	40 μM
	3.60	5.79	1.05	0.99	3.03	1.81	5.20	-0.46	-0.50	-1.15
	3.70	8.31	3.01	4.25	7.14	8.04	4.82	-0.85	2.40	2.25
	6.64	9.92	3.89	4.44	6.43	10.52	3.72	-0.75	2.11	2.66
	10.53	14.61	7.80	8.90	8.11	9.71	1.89	3.37	3.92	4.54
	9.80	15.26	3.73	6.72	7.28	9.16	1.35	5.50	3.05	4.35
	9.34	15.04	6.06	8.22	7.36	8.46	3.69	5.83	3.00	5.19
	1.80	4.28	-0.76	0.21	0.61	5.48	0.81	2.94	0.95	0.41
	2.62	5.36	0.89	1.81	0.24	3.05	0.73	2.38	0.74	1.52
	3.68	9.71	1.69	3.06	2.45	6.41	0.70	1.88	0.71	-0.27
AVG	5.75	9.81	3.04	4.29	4.74	6.96	2.55	2.20	1.82	2.17
STDEV	3.38	4.32	2.69	3.12	3.14	3.02	1.82	2.53	1.43	2.24
SEM	1.95	2.49	1.55	1.80	1.81	1.74	1.05	1.46	0.82	1.29

PC3	2		3		4		5		6	
	20 μM	40 μM	20 μM	40 μM	20 μM	40 μM	20 μM	40 μM	20 μM	40 μM
	-0.27	-0.12	1.19	4.65	4.06	3.30	0.10	-1.66	4.96	-1.04
	0.05	0.42	1.54	2.80	2.49	1.79	0.17	-1.48	5.04	-0.79
	1.90	1.07	2.70	3.53	2.43	2.01	0.02	-0.56	4.87	-0.42
	2.20	2.02	5.37	10.46	0.10	-1.06	0.37	1.73	0.96	0.03
	1.35	0.63	7.62	8.30	0.70	0.01	-1.99	-0.15	-0.16	-0.54
	1.41	1.71	5.53	7.30	1.41	-0.93	-2.54	-2.47	-0.48	-1.35
	1.30	7.53	0.11	0.23	0.19	0.08	1.46	0.73	2.36	2.91
	-0.32	8.18	0.85	0.80	-0.38	-0.12	0.75	0.88	2.68	2.36
	1.30	9.93	1.04	0.53	0.06	0.62	-0.44	-0.24	3.39	0.46
AVG	0.99	3.49	2.88	4.29	1.23	0.63	-0.23	-0.36	2.62	0.18
STDEV	0.93	3.90	2.63	3.69	1.49	1.45	1.27	1.35	2.16	1.50
SEM	0.54	2.25	1.52	2.13	0.86	0.84	0.74	0.78	1.25	0.87

S2.4.4. Determination of ERK1/2 Phosphorylation, PKCα and –δ and PSMA expression by Immunoblotting

The PSA/PSMA-positive 22Rv1 and PSA/PSMA-negative DU145 human PC cells were seeded onto 6-well plates at a density of 4.0×10^5 cells/well. Then, 20-24 h after seeding, the medium was changed to serum free RPMI1640. After a 2-h serum starvation, the cells were treated with the test compounds for 30 min, washed twice with ice-cold PBS, and harvested in ice-cold lysis buffer (1 mM EDTA, 150 mM NaCl, 0.25% NP-40, 1% Triton X-100, 10 mM Tris/HCl, pH 6.8) supplemented with protease and phosphatase inhibitors (Complete and PHOStop, respectively; Roche, Mannheim, Germany). Lysates were centrifuged (13,000 *g*, 4 min, 4 °C) and the supernatants collected. Equal amounts of protein (10 µg) were subjected to reducing SDS-PAGE and transferred to poly(vinylidene difluoride) membrane.

After blocking the membranes with 5% milk in 0.1% Tween 20 in Tris-buffered saline (TBST) for 1 h at room temperature the cells were incubated overnight at 4 °C in a shaker with primary antibodies against p44/42 MAPK (Erk1/2) (#9102; 1:2000, Cell Signaling Technology, Danvers, MA, USA), phospho-p44/42 MAPK (Erk1/2) (#9101; 1:2000, Cell Signaling Technology, Danvers, MA, USA) and GAPDH (sc47724, 1:2000, Santa Cruz Biotechnology, Dallas, TX, USA) in blocking buffer. For determining the PKC and PSMA protein levels in 22Rv1 cells, all primary antibodies were from Abcam PKCα (ab 32376), PKCδ (ab 182126) and PSMA (ab 76104) and were used as 1:1000 dilution. The experiments were repeated three times with two wells per condition in each experiment. The following day, the membranes were washed with TBST and incubated with blocking buffer containing HRP-linked secondary antibody (goat anti-rabbit, #170-6515; Bio-Rad, CA, USA or antimouse IgG #7076S; Cell Signaling Technology) for 1 h at RT. Secondary antibodies were detected with chemiluminescent substrate (SuperSignal West Pico, #34080; Thermo Fisher) utilizing ChemiDoc XRS+ imaging System (Bio-Rad Laboratories, Hercules, CA, USA). Quantification was carried out by measuring the optical densities of the immunoreactive bands using ImageJ software (https://imagej.net/Downloads). The optical densities were always first normalized to GAPDH from the same sample and then to the corresponding control (cells treated with the vehicle only) on the same membrane.

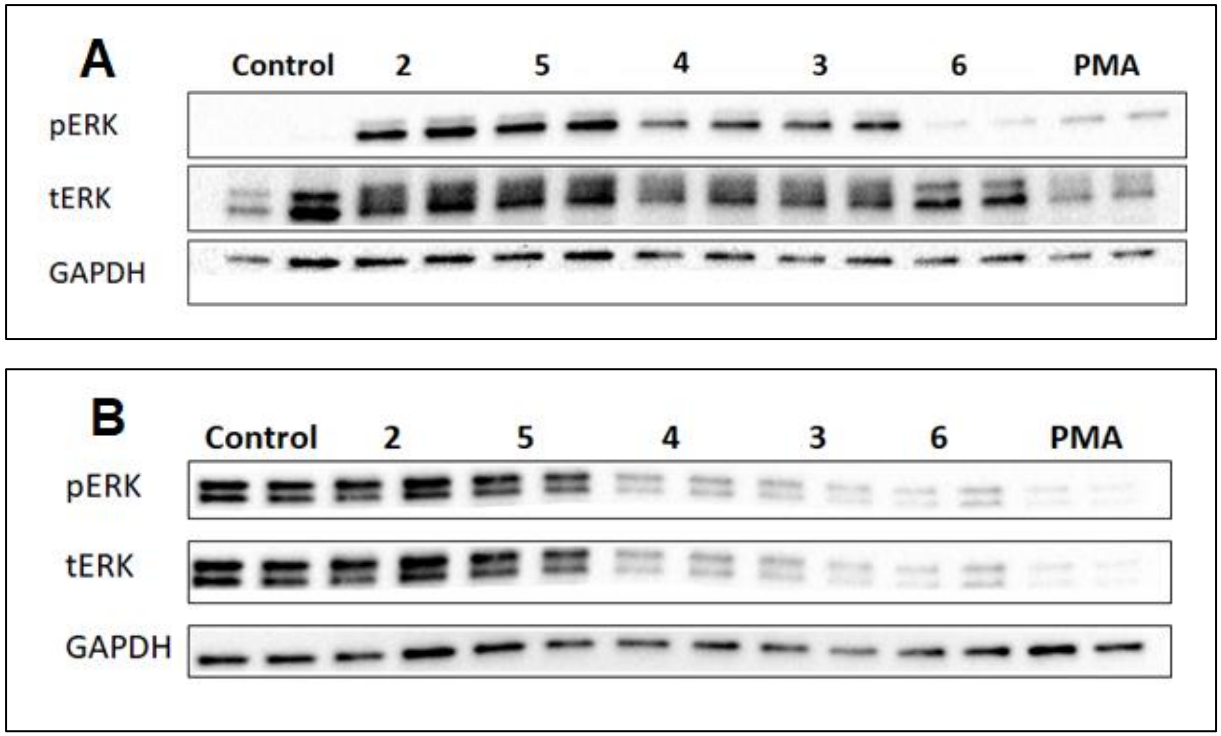


Fig. S3 Representative blot images from a single ERK phosphorylation experiment, with two parallel samples for each condition on 22Rv1 (A) and DU145 cells (B).

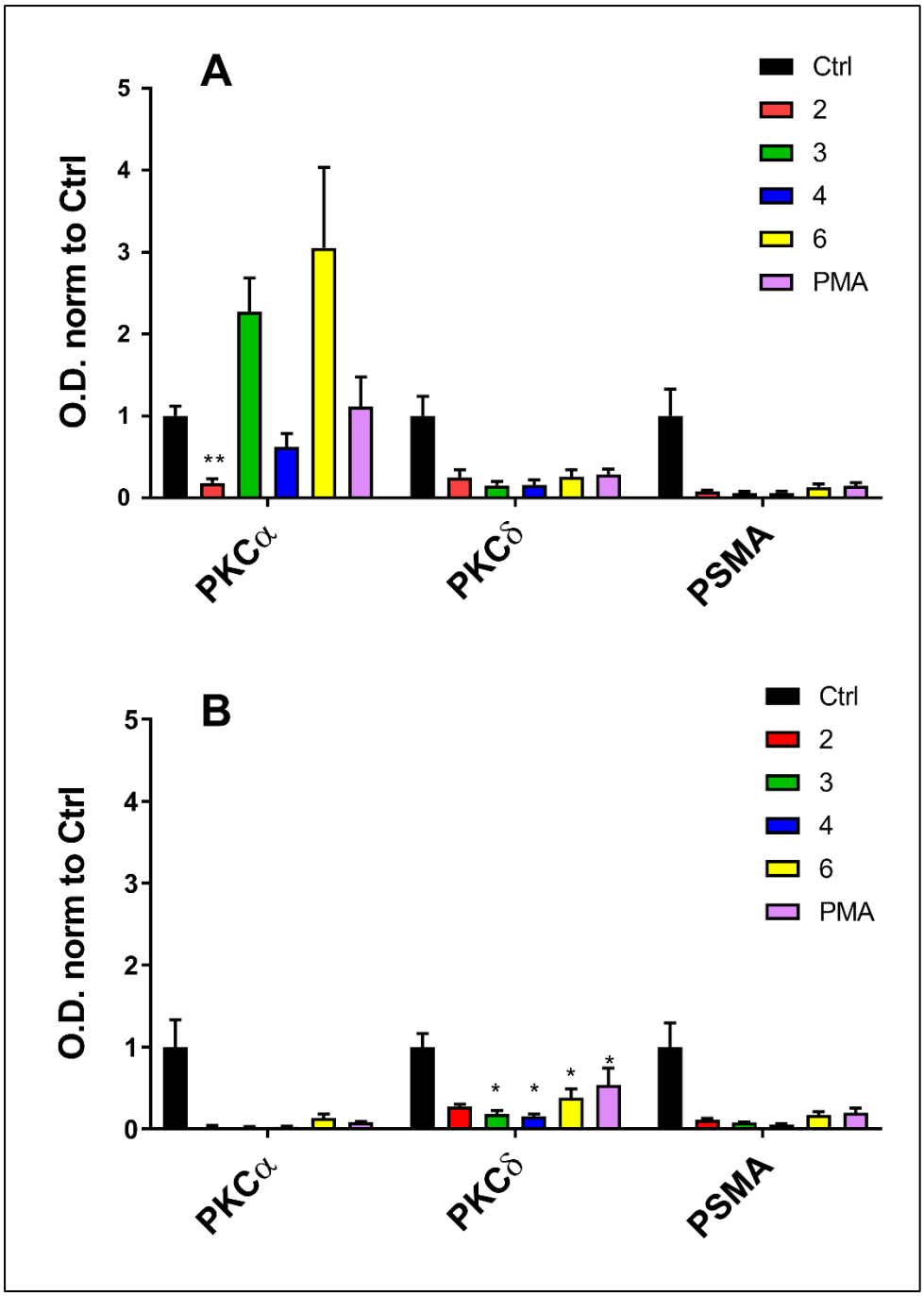


Fig. S4 PKCα, -δ and PSMA expression in 22Rv1 cell line after 24 h (A) and 72 h (B) exposure to the test compounds 2, 3, 4, 6 and PMA. Results are presented as mean + SEM (N = 3; *P < 0.05 vs ctrl, **P <0.01 Welch's t-test).

Table S3 PKCα, -δ and PSMA expression in 22Rv1 cell line after 24 h and 72 h exposure numerical data.

24 h	PKCα	SEM	PKCδ	SEM	PSMA	SEM
Ctrl	1.00	0.12	1.00	0.24	1.00	0.32
2	0.18	0.05	0.24	0.10	0.07	0.02
3	2.27	0.41	0.15	0.05	0.06	0.02
4	0.62	0.17	0.15	0.06	0.06	0.02
6	3.05	0.98	0.26	0.09	0.13	0.04
PMA	1.11	0.36	0.28	0.07	0.15	0.04

72 h	PKC**α**	SEM	PKC**δ**	SEM	PSMA	SEM
Ctrl	1.00	0.34	1.00	0.17	1.00	0.30
2	0.02	0.02	0.28	0.03	0.11	0.02
3	0.02	0.01	0.19	0.04	0.08	0.01
4	0.03	0.01	0.16	0.03	0.06	0.01
6	0.14	0.05	0.38	0.11	0.17	0.05
PMA	0.09	0.00	0.54	0.20	0.20	0.06

Table S4 MTT assay numerical data presented as averages (±SEM) from three individual experiments and three parallel wells in each experiment. (% of control).

					2
		LNcaP	22Rv1	DU145	PC3
	0 µM	100.00	100.00	100.00	100.00
	1 µM	71.18	73.49	86.99	103.34
AVG	10 µM	84.34	70.09	80.84	106.47
	20 µM	78.41	44.42	65.99	80.77
	40 µM	61.05	8.38	40.97	27.67
		3.56	6.78	5.98	10.53
		1.92	6.99	4.08	8.96
SEM		4.48	7.82	4.17	13.67
		7.23	19.09	14.42	8.71
		4.17	3.06	13.97	16.09

					3
		LNcaP	22Rv1	DU145	PC3
	0 µM	100.00	100.00	100.00	100.00
	1 µM	80.75	93.44	110.00	89.33
AVG	10 µM	84.81	78.92	140.10	99.08
	20 µM	83.00	87.65	131.54	99.97
	40 µM	86.36	82.72	153.41	102.34
		3.56	6.78	5.98	10.53
		8.16	11.51	15.62	10.29
SEM		10.31	8.76	29.90	11.13
		9.01	13.88	32.32	12.50
		9.08	13.21	38.53	13.33

					4
		LNcaP	22Rv1	DU145	PC3
	0 µM	100.00	100.00	100.00	100.00
	1 µM	78.55	82.06	97.46	78.46
AVG	10 µM	85.31	74.33	92.48	76.68
	20 µM	82.67	54.18	83.26	73.37
	40 µM	82.48	26.65	74.14	16.72
		3.56	6.78	5.98	9.12
		8.72	11.50	6.97	9.29
SEM		7.10	6.53	4.70	9.03
		6.75	7.80	5.05	11.18
		4.69	13.40	3.43	10.85

					5
		LNcaP	22Rv1	DU145	PC3
AVG	0 µM	100.00	100.00	100.00	100.00
	1 µM	61.95	104.72	89.00	72.30

	10 µM	50.60	90.83	83.41	80.45
	20 µM	40.50	87.51	85.04	84.41
	40 µM	41.00	74.68	70.90	65.97
		3.56	6.78	5.98	10.53
		13.35	14.13	5.58	5.94
SEM		13.15	8.89	3.23	6.10
		2.48	9.17	8.81	8.07
		4.78	9.69	2.40	10.97
					6
		LNcaP	22Rv1	DU145	PC3
	0 µM	100.00	100.00	100.00	100.00
	1 µM	91.24	96.04	104.25	91.66
AVG	10 µM	70.40	64.47	77.88	77.85
	20 µM	70.79	55.00	69.78	76.33
	40 µM	65.84	50.29	63.31	12.91
		3.56	6.78	5.98	10.53
		7.03	8.04	16.02	6.59
SEM		4.79	13.71	17.84	8.47
		4.14	16.25	20.44	9.69
		4.82	19.33	21.61	2.01
					Tg3
		LNcaP	22Rv1	DU145	PC3
	0 µM	100.00	100.00	100.00	100.00
	1 µM	71.05	60.50	82.99	38.28
AVG	10 µM	26.45	31.11	35.13	19.38
	20 µM	10.56	28.56	31.75	18.16
	40 µM	3.61	26.50	19.69	15.57
		3.56	6.78	5.98	10.53
		8.64	8.60	1.96	4.61
SEM		4.57	7.77	2.26	1.59
		3.27	7.04	1.76	3.66
		1.47	7.47	1.94	3.03
					Tg6
		LNcaP	22Rv1	DU145	PC3
	0 µM	100.00	100.00	100.00	100.00
	1 µM	94.64	97.97	96.92	94.45
AVG	10 µM	71.19	69.27	86.42	67.89
	20 µM	67.54	56.47	78.88	48.25
	40 µM	35.45	40.39	67.07	32.44

		3.56	6.78	5.98	10.53
		4.72	4.44	4.57	9.45
SEM		4.69	6.69	3.95	10.73
		5.03	8.53	1.65	7.35
		15.89	9.28	4.01	3.87

Table S5 MTT assay numerical data presented as averages from three individual experiments and three parallel wells in each experiment without and with 1 µM PKC inhibitor Gö6983 (% of control).

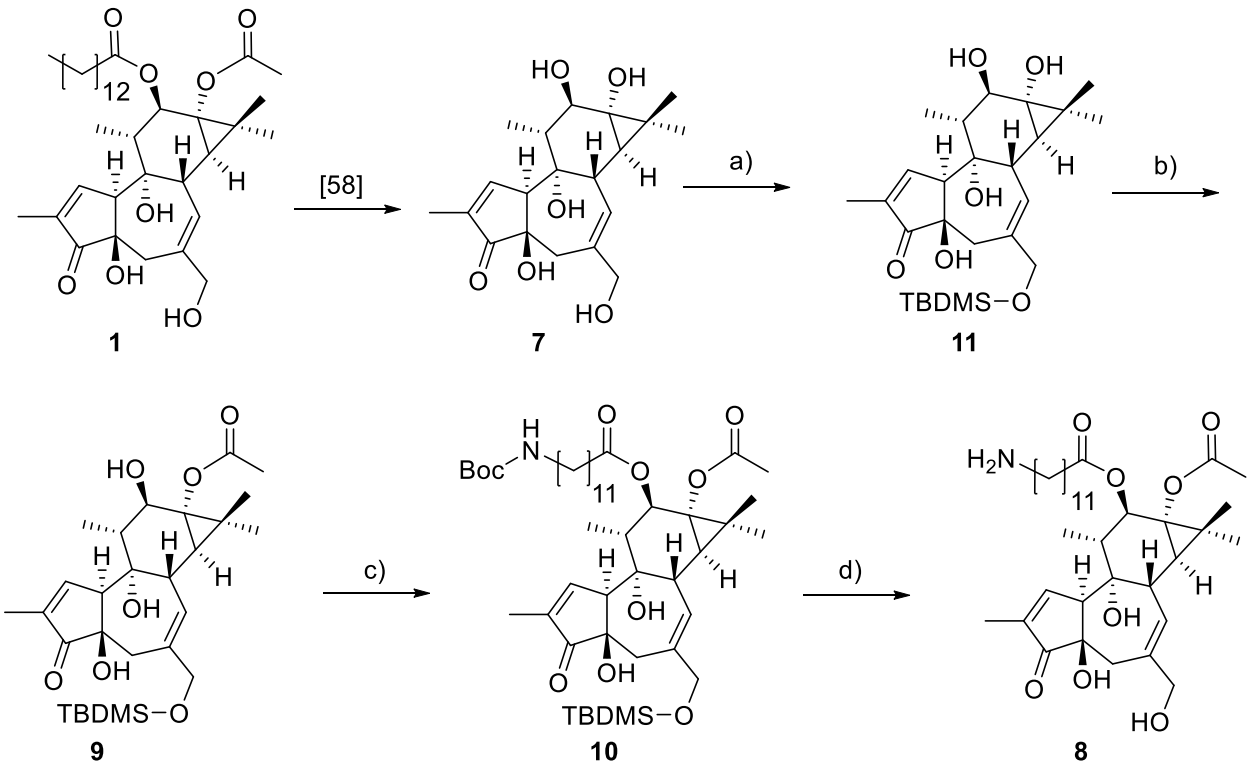
		LNcaP	22Rv1	DU145	PC3
2	20 µM	78.41	44.42	65.99	80.77
	20 µM + Gö6983	90.02	36.69	78.84	114.92
3	20 µM	83.00	87.65	136.56	99.97
	20 µM + Gö6983	92.56	121.47	111.94	110.45
4	20 µM	82.67	54.18	83.26	73.37
	20 µM + Gö6983	101.91	69.75	97.71	95.70
5	20 µM	40.50	87.51	85.04	84.41
	20 µM + Gö6983	74.31	111.03	96.37	105.99
6	20 µM	70.79	55.00	69.78	76.33
	20 µM + Gö6983	94.18	61.22	66.31	102.79
Tg3	20 µM	10.56	28.56	28.28	18.16
	20 µM + Gö6983	15.50	24.56	24.00	19.02
Tg6	20 µM	67.54	56.47	74.70	48.25
	20 µM + Gö6983	55.04	53.68	76.24	53.08
PMA	100 nM	52.72	96.95	85.81	89.22
	100 nM + Gö6983	93.65	108.46	103.73	79.41
Gö6983	1 µM	90.98	99.67	99.20	100.15

S2.5. Synthesis of phorbol derivatives

*S2.5.1. Isolation of the starting material 4β-phorbol (**7**)*

The starting 4β-phorbol (**7**) was obtained from seeds of *C. tiglium* by extraction, fractionation and solvolysis as reported.[1] All NMR or HRMS data correspond to those already published.[5]

In Scheme S1 PMA (**1**) is depicted as a representative of phorbol esters. The isolated fraction consists of a number of phorbol esters acylated at O-12 and O-13 and in addition some also acylated at O-20.[1, 6] Solvolysis of the mixture of phorbol esters provided 4β-phorbol (**7**) as previously described.[1] Protection of the primary alcohol at C-20 and selective acetylation at O-13 gave intermediate **9**, which after esterification at *O*-12 with Boc-protected 12-Aminododecanoic acid and subsequent removal of the Boc group yielded starting material **8** (Scheme S6).



Scheme S6. Synthesis of 12-*O*-(12-Aminododecanoyl)-13-*O*-acetyl-4β-phorbol **8**. Reagents and conditions: (**a**) TBDMS-Cl, DMAP, imidazole, DMF, 23 °C, 2 h (53%); (**b**) DMAP, Et_3N, 10% Ac_2O in DCM, 0 °C to 23 °C, 75 min (85%); (**c**) Boc-12-aminododecanoic acid, DMAP, EDC, DCM, 23 °C, 20 h (94%); (**d**) TFA–DCM (2:1), 30 min, 23 °C (91%).

*S2.5.2. 20-O-TBDMS-4β-phorbol (**11**)*

4β-Phorbol (**7**; 1.19 g; 3.27 mmol) was dissolved in DMF (20 mL), and while stirring on ice DMAP (80 mg; 0.65 mmol), imidazole (675 mg; 9.92 mmol) and TBDMSCl (739 mg; 4.90 mmol) were added. The reaction mixture was left to warm up to 23 °C, and after additional 4 h more TBDMS (492 mg; 3.27 mmol) was added to convert residual amounts of the starting 4β-phorbol. After additional 2 h the solvent was removed from the reaction mixture, and then the residue was fractionated on a silica column (eluted with DCM–MeOH 20:1) to give **11** as a white crystalline powder (1.34 g; 85%). ^{1}H NMR (600 MHz, methanol-d_4) δ ppm 7.62 (dd, J = 2.1, 1.3 Hz, 1 H, H-1), 5.62 (d, J = 4.4 Hz, 1 H, H-7), 4.05 (d, J = 10.0 Hz, 1 H, H-12), 4.06 (s, 2 H, H-20), 3.13 (t, J = 5.5 Hz, 1 H, H-8), 3.09 (t, J = 2.7 Hz, 1 H, H-10), 2.50 (d, J = 19.1 Hz, 1 H, H_a-5), 2.45 (d, J = 19.1 Hz, 1 H, H_a-5), 1.94–1.99 (m, 1 H, H-11), 1.75 (dd, J = 2.8, 1.3 Hz, 3 H, H-19), 1.28 (s, 3 H, H-16), 1.17 (s, 3 H, H-17), 1.08 (d, J = 6.6 Hz, 3 H, H-18), 0.90 (s, 9H, TBDMS 3 × CH_3), 0.64 (d, J = 5.4 Hz, 1 H, H-14), 0.00 (s, 3H, Si-CH_3), -0.01 (s, 3H, Si-CH_3). ^{13}C NMR (150 MHz, methanol-d_4) δ ppm 209.37 (1 C, C-3), 159.79 (1 C, C-1), 140.21 (1 C, C-6), 132.83 (1 C, C-2), 129.32 (1 C, C-7), 80.71 (1 C, C-12), 78.26 (1 C, C-9), 73.57 (1 C, C-4), 67.79 (1 C, C-20), 61.86 (1 C, C-13), 57.40 (1 C, C-10), 44.79 (1 C, C-11), 38.95 (1 C, C-8), 36.90 (1 C, C-5), 36.02 (1 C, C-14), 25.73 (1 C, C-15), 24.98 (3 C, TBDMS 3 × CH_3) 22.71 (1 C, C-17), 17.75 (1 C, Si-C-$(CH_3)_3$) 16.44 (1 C, C-16), 14.03 (1 C, C-18), 8.84 (1 C, C-19), -6.55 (2 C, Si-$(CH_3)_2$).

*S2.5.3. 13-O-Ac-20-O-TBDMS-4β-phorbol (**9**)*

After dissolving 20-*O*-TBDMS**-**4β-phorbol (**11**; 412 mg; 0.86 mmol) in DCM (1.5 mL) and THF (1.5 mL), Et_3N (0.23 mL; 1.72 mmol) and DMAP (11 mg; 0.09 mmol) were added, followed by slow addition of a 10% solution of Ac_2O in DCM (0.9 mL; 0.86 mmol) while stirring on ice. Shortly after addition of the Ac_2O solution the ice bath was removed, and then the reaction mixture was allowed to warm to 23 °C for 1.5 h. The solvent was evaporated, the residue was dissolved in DCM and washed with brine and a saturated solution of NH_4Cl in water. The organic phase was dried over Na_2SO_4, evaporated, and the resulting residue fractionated on silica gel (200 mL silica gel, eluting with heptane–EtOAc 2:1 and 3:2) to give 12,13-di-*O*-Ac-20-*O*-TBDMS-4β-phorbol (76 mg) and the desired product **9** as a yellow powder (379 mg; 85%). ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 7.57 (dd, J = 2.1, 1.3 Hz, 1 H, H-1), 5.61 (d, J = 4.4 Hz, 1 H, H-7), 4.0 (d, J = 10.0 Hz, 1 H, H-12), 4.02 (s, 2 H, H-20), 3.15 (t, J = 5.5 Hz, 1 H, H-8), 3.13 (t, J = 2.7 Hz, 1 H, H-10), 2.47 (d, J = 19.1 Hz, 1 H, H_a-5), 2.37 (d, J = 19.1 Hz, 1 H, H_b-5), 2.13 (s, 3 H, H-2Ac), 2.05-1.99 (m, 1 H, H-11), 1.77 (dd, J = 2.8, 1.3 Hz, 3 H, H-19), 1.25 (s, 3 H, H-16), 1.22 (s, 3 H, H-17), 1.04 (d, J = 6.6 Hz, 3 H, H-18), 1.03 (d, J = 5.4 Hz, 1 H, H-14), 0.90 (s, 9H, TBDMS 3 × CH_3), 0.07 (s, 3H, Si-CH_3), 0.06 (s, 3H, Si-CH_3). ^{13}C NMR

(150 MHz, $CDCl_3$) δ ppm 208.87 (1 C, C-3), 174.09 (1C, C-1 Ac), 160.30 (1 C, C-1), 140.66 (1 C, C-6), 132.95 (1 C, C-2), 127.51(1 C, C-7), 78.26 (1 C, C-9), 77.48 (1 C, C-12), 73.53 (1 C, C-4), 68.33 (1 C, C-13) 67.86 (1 C, C-20), 56.90 (1 C, C-10), 44.90 (1 C, C-11), 39.02 (1 C, C-8), 38.35 (1 C, C-5), 35.42 (1 C, C-14), 26.68 (1 C, C-15), 25.94 (3 C, TBDMS 3 $\times$ CH_3), 23.62 (1 C, C-17), 21.07 (1 C, C-2 Ac), 18.38 (1 C, Si-C-$(CH_3)_3$), 16.92 (1 C, C-16), 15.10 (1 C, C-18), 10.10 (1 C, C-19), -6.55 (2 C, Si-$(CH_3)_2$).

*S2.5.4. 12-O-(Boc-12-Aminododecanoyl)-13-O-Ac-20-O-TBDMS-4β-phorbol (**10**)*

Compound **9** (159 mg; 0.300 mmol) was dissolved in DCM (4 mL) and then DMAP (56 mg; 0.46 mmol), EDC (86 mg; 0.45 mmol) and Boc-12-aminododecanoate (142 mg; 0.45 mmol) were added. The mixture was stirred for 20 h at 23 °C, the solvent was evaporated, and the residue purified on a silica column (heptane–EtOAc 3:1) to give product **10** as a yellow powder (250 mg; 94%). ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 7.60 (dd, *J* = 2.1, 1.3 Hz, 1 H, H-1), 5.63 (d, *J* = 4.4 Hz, 1 H, H-7), 5.42 (d, *J* = 10.0 Hz, 1 H, H-12), 4.02 (s, 2 H, H-20), 3.25 (t, *J* = 2.7 Hz, 1 H, H-10), 3.19 (t, *J* = 5.5 Hz, 1 H, H-8), 3.10 (d, *J* = 5.9 Hz, 2 H, H´-12), 2.50 (d, *J* = 19.1 Hz, 1 H, H_a-5), 2.41 (d, *J* = 19.1 Hz, 1 H, H_b-5), 2.32 (m, 2 H, H´-2), 2.18 - 2.13 (m, 1 H, H-11), 2.10 (s, 3 H, H-2 Ac), 1.77 (dd, *J* = 2.8, 1.3 Hz, 3 H, H-19), 1.64 (quin, *J* = 7.3, 2 H, H´-3), 1.47 (m, 2 H, H´-11), 1.45 (s, 9 H, Boc), 1.35 - 1.26 (m, 14 H, H´-10 $\rightarrow$ H´-4), 1.24 (s, 3 H, H-16), 1.21 (s, 3 H, H-17), 1.07 (d, *J* = 5.4 Hz, 1 H, H-14), 0.90 (d, *J* = 6.6 Hz, 3 H, H-18), 0.89 (s, 9 H, TBDMS 3 $\times$ CH_3), 0.07 (s, 3 H, Si-CH_3), 0.06 (s, 3 H, Si-CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ ppm 208.97 (1 C, C-3), 173.69 (1 C, C´-1), 173.58 (1 C, C-1 Ac), 160.77 (1 C, C-1), 155.97 (1 C, Boc carbonyl) 140.08 (1 C, C-6), 132.61 (1 C, C-2), 128.05 (1 C, C-7), 77.97 (1 C, C-9), 76.80 (1 C, Boc C-$(CH_3)_3$) 76.68 (1 C, C-12), 73.80 (1 C, C-4), 68.01 (1 C, C-20), 65.71 (1 C, C-13), 56.13 (1 C, C-10), 42.91 (1 C, C-11), 40.61 (1 C, C´-12), 39.10 (1 C, C-8), 38.29 (1 C, C-5), 36.26 (1 C, C-14), 34.57 (1 C, C´-2), 30.05 (1 C, C´-11), 29.50-28.43 (7 C, C´4 - C´10), 28.43 (3 C, Boc 3 $\times$ CH_3), 25.94 (3 C,TBDMS 3 $\times$ CH_3), 25.54 (1 C, C-15), 25.16 (1 C, C´-3), 23.85 (1 C, C-17), 21.10 (1C, C-2 Ac), 18.38 (1 C, Si-C-$(CH_3)_3$), 16.77 (1 C, C-16), 14.38 (1 C, C-18), 10.08 (1 C, C-19), -6.27 (2 C, Si-$(CH_3)_2$).

*S2.5.5. 12-O-(12-Aminododecanoyl)-13-O-Ac-4β-phorbol (**8**)*

To a solution of compound **10** (170 mg; 0.21 mmol) in DCM (3 mL) was added TFA (3 mL) under stirring on ice. Then the reaction mixture was allowed to warm to room temperature during 2 h. On TLC the product was visualized by spraying with ninhydrin in *n*-butanol and visualized by heating with a heat gun. The reaction mixture was concentrated, and the resulting residue was fractionated on a silica column (DCM–MeOH 9:1) to give the free amine **8** as a yellow amorphous powder (114 mg; 91%). Analytical UHPLC with the gradient 20% $\rightarrow$ 100%

B during 10 min: retention time of 5.7 min; revealed a purity of >96%. ^{1}H NMR (600 MHz, methanol-d_4) δ ppm 7.56 (dd, J = 2.1, 1.3 Hz, 1 H, H-1), 5.64 (d, J = 4.4 Hz, 1 H, H-7), 5.46 (d, J = 10.0 Hz, 1 H, H-12), 3.99–3.92 (dd, J = 12.8, 9.8 Hz, 2 H, H-20), 3.31 (t, J = 5.5 Hz, 1 H, H-8), 3.18 (t, J = 2.7 Hz, 1 H, H-10), 2.9, (t, J = 7.7 Hz, 2 H, H´-12), 2.56 (d, J = 19.1Hz, 1 H, H_a-5), 2.50 (d, J = 19.1 Hz, 1 H, H_b-5), 2.42-2.32 (m, 2 H, H´-2), 2.26 - 2.20 (m, 1 H, H-11), 2.08 (s, 3 H, H-2 Ac), 1.76 (dd, J = 2.8, 1.3 Hz, 3 H, H-19), 1.69 - 1.63 (m, 4 H, H´-3, H´-11), 1.44 - 1.31, (m, 14 H, H´-10 $\rightarrow$ H´-4), 1.27 (s, 3 H, H-16), 1.23 (s, 3 H, H-17), 1.17 (d, J = 5.4 Hz, 1 H, H-14), 0.91 (d, J = 6.6 Hz, 3 H, H-18). ^{13}C NMR (150 MHz, methanol-d_4) δ ppm 208.90 (1 C, C-3), 174.21 (1 C, C´-1), 173.69 (1 C, C-1 Ac), 159.13 (1 C, C-1), 141.14 (1 C, C-6), 133.18 (1 C, C-2), 127.90 (1 C, C-7), 78.38 (1 C, C-9), 76.83 (1 C, C-12), 73.32 (1 C, C-4), 66.58 (1 C, C-20), 65.73 (1 C, C-13), 55.94 (1 C, C-10), 53.41 (1 C, C-13), 42.85 (1 C, C-11), 39.36 (1 C, C´-12), 38.61 (1 C, C-8), 37.07 (1 C, C-5), 35.64 (1 C, C-14), 33.95 (1 C, C´-2), 29.20-28.43 (7 C, C´4 - C´10), 27.17 (1 C, C´-3), 26.05 (1 C, C-15), 24.84 (1 C, C´-11), 22.72 (1 C, C-17), 19.68 (1C, C-2 Ac), 15.96 (1 C, C-16), 13.45 (1 C, C-18), 8.64 (1 C, C-19). HRMS-ESI: $[C_{34}H_{53}NO_8+H]^+$ *m/z:* 604.3849. Found 604.3845.

*S2.5.6. 12-O-(β-Asp-12-Aminododecanoyl)-13-O-Ac-4β-phorbol (**3**)*

To a solution of 12-Aminododecanoic acid (4.71 g; 21.9 mmol) in 90 mL acetone and 90 mL H_2O were added Fmoc-OSu (6.55 g; 21.9 mmol) and $NaHCO_3$ (2.39 g; 28.5 mmol) and the reaction was stirred at 23 °C for 20 h. The reaction mixture was acidified with conc. HCl until pH 4-5, and the resulting precipitate was extracted with EtOAc (3 × 50 mL). The combined organic phases were dried with Na_2SO_4, filtered, and concentrated. The crude product was purified on a silica column (hexane–EtOAc 3:1) to provide Fmoc-12-aminododecanoic acid (4.20 g; 43%) as a white solid. ^{1}H NMR and ^{13}C NMR were as reported [7]. 2-CTC resin (2.58 g; loading: 1.6 mmol/g) was swelled in DCM (5 mL) in a teflon reaction vessel fitted with a polypropylene filter, and a solution of Fmoc-12-aminododecanoic acid (0.60 g; 1.37 mmol) and DIPEA (2.38 mL; 13.7 mmol) in DCM (3 mL) was added to the resin. The mixture was shaken for 3 h at 23 °C. The resin was drained and washed twice with DCM followed by capping with DIPEA–MeOH–DCM 5:15:80 (8 mL; 2 × 5 min). The resin was washed successively with DMF, MeOH and DCM (each 3 times with 8 mL for 3 min), and then residual solvent was removed on a freeze-dryer. Test cleavage of the dry preloaded resin with a 20% solution of HFIP in DCM showed a loading of approx. 0.48 mmol/g.

The Fmoc protecting group was removed with a 20% solution of piperidine in DMF (8 mL; 2 × 20 min) followed by washing with DMF, MeOH and DCM (each 3 times as above). A solution of Fmoc-Asp-O*t*Bu (587 mg; 1.42 mmol), PyBOP (743 mg; 1.42 mmol) and DIPEA (0.25 mL; 2.62 mmol) dissolved in DMF (6 mL) was added to the resin-bound 12-Aminododecanoic acid

(1.00 g, 0.48 mmol loading). The mixture was shaken for 4 h at 23 °C, an then a final standard washing (as above) of the resin performed, and finally the product was cleaved from the resin with a 20% solution of HFIP in DCM (3 × 6 mL; each time for 30 min). The eluate was concentrated to give Fmoc-Asp-O*t*Bu-12-aminododecanoate (140 mg; 0.23 mmol; 93%) as colorless amorphous solid.

To a solution of Fmoc-Asp-O*t*Bu-12-aminododecanoic acid (140 mg; 0.23 mmol) in DCM (3 mL) was added 13-*O*-Ac-20-*O*-TBDMS-4β-phorbol (**9**; 103 mg; 0.20 mmol), 10 mol % DMAP (2.5 mg; 0.02 mmol) and EDC (56 mg; 0.29 mmol). To avoid loss of the Fmoc group DMAP (5 mg; 0.04 mmol) was added four times with 1 h intervals. The reaction was stopped after 25 h. The reaction mixture was concentrated, and the residue purified on silica column (toluene–EtOAc 4:1) to provide 12-*O*-(Fmoc-Asp-OtBu-12-aminododecanoyl)-13-*O*-Ac-20-TBDMS-4β-phorbol (**14**; 92 mg; 42%).

To a solution of compound **14** (92 mg; 0.08 mmol) in DCM (2 mL) and THF (3 mL) added a few drops of H_2O were added, the and the reaction was stirred at 23 °C for 1 h. and TFA and DCM were removed by evaporation and DCM (4 mL) was added followed by addition of Et_2NH (1 mL). The reaction was stirred at 23 °C for 1 h. The reaction mixture was then concentrated. The residue was purified by preparative HPLC with the gradient 50% → 100% B during 20 min to give 12-*O*-(Asp-12-aminododecanoyl)-13-*O*-Ac-20-TBDMS-4β-phorbol **3** as white powder (37 mg; 62%). Analytical UHPLC with gradient 50% → 100% B during 10 min: retention time 2.2 min; purity ≥99%. ^{1}H NMR (600 MHz, methanol-d_4) δ ppm 7.57 (s, 1 H, C-3), 5.64 (d, J = 4.4 Hz, 1 H, H-7), 5.47 (d, J = 10.0 Hz, 1 H, H-12), 4.23 (dd, J = 7.1, 4.4 Hz, 1 H, α-H Asp), 3.96 (dd, J = 12.8, 9.8 Hz, 2 H, H-20), 3.31 (m, 1 H, H-8), 3.22 (m, 1 H, H-10), 3.19 - 3.16 (m, 2 H, H´-12), 2.96 - 2.81 (m, 2 H, β-H Asp), 2.54 (d, J = 19.1 Hz, 1 H, H_a-5), 2.50 (d, J = 19.1 Hz, 1 H, H_b-5), 2.44 - 2.30 (m, 2 H, H´-2), 2.24 (m, 1 H, H-11), 2.08 (s, 3 H, H-2 Ac), 1.76 (dd, J = 2.8, 1.3 Hz, 3 H, H-19), 1.70 - 1.62 (m, 2 H, H´-3), 1.52 (m, 2 H, H´-11), 1.42 - 1.30 (m, 14 H, H´-10 → H´-4), 1.27 (s, 3 H, H-16), 1.23 (s, 3 H, H-17), 1.17 (d, J = 5.4 Hz, 1 H, H-14), 0.91 (d, J = 6.6 Hz, 3 H, H-18). ^{13}C NMR (150 MHz, methanol-d_4) δ ppm 208.91 (1 C, C-3), 174.23 (1 C, C´-1), 173.89 (1 C, C-1 Ac), 169.65 (1 C, C-4 Asp), 169.49 (1 C, C-1 Asp) 159.13 (1 C, C-1), 141.44 (1 C, C-6), 133.19 (1 C, C-2), 127.89 (1 C, C-7), 78.37 (1 C, C-9), 76.85 (1 C, C-12), 73.32 (1 C, C-4), 66.57 (1 C, C-20), 65.73 (1 C, C-13), 55.94 (1 C, C-10), 49.69 (1 C, C-3 Asp), 42.85 (1 C, C-11), 39.14 (1 C, C´-12), 38.61 (1 C, C-8), 37.07 (1 C, C-5), 35.63 (1 C, C-14), 33.95 (1 C, C´-2), 33.81 (1 C, C-2 Asp), 29.24-28.65 (7 C, C´-4 - C´-9 + C´-11), 26.59 (1 C, C´-10), 25.78 (1 C, C-15) 24.83 (1 C, C´-3), 22.70 (1 C, C-17), 19.66 (1C, C-2 Ac), 15.98 (1 C, C-16), 13.43 (1 C, C-18), 8.81 (1 C, C-19). HRMS-ESI: $[C_{38}H_{58}N_2O_{11}+H]^+$ *m/z:* 719.4119. Found 719.4112.

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Method Filename : 50_100B_10min_crude2014.lcm
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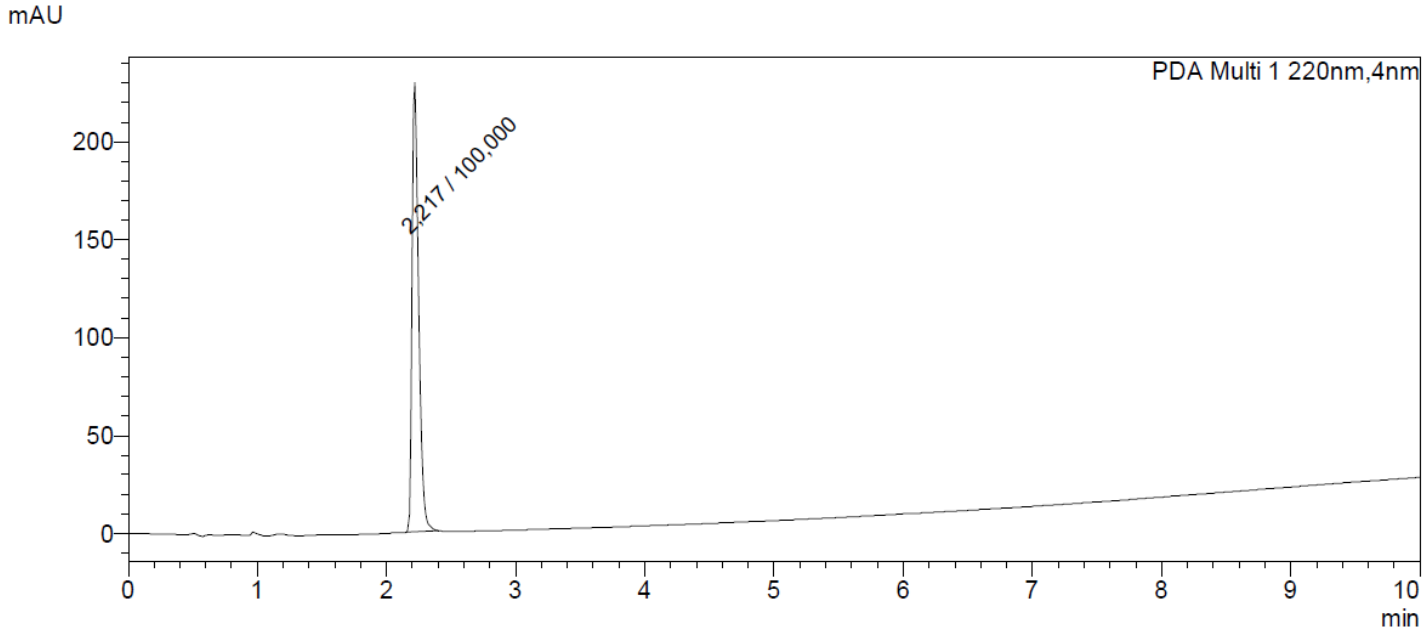
Vial # : 1-63
Injection Volume : 5 uL
Date Acquired : 6/8/2017

Complete Chromatogram



PDA Ch1 220nm

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Fig.S6: Compound 3 analytical HPLC

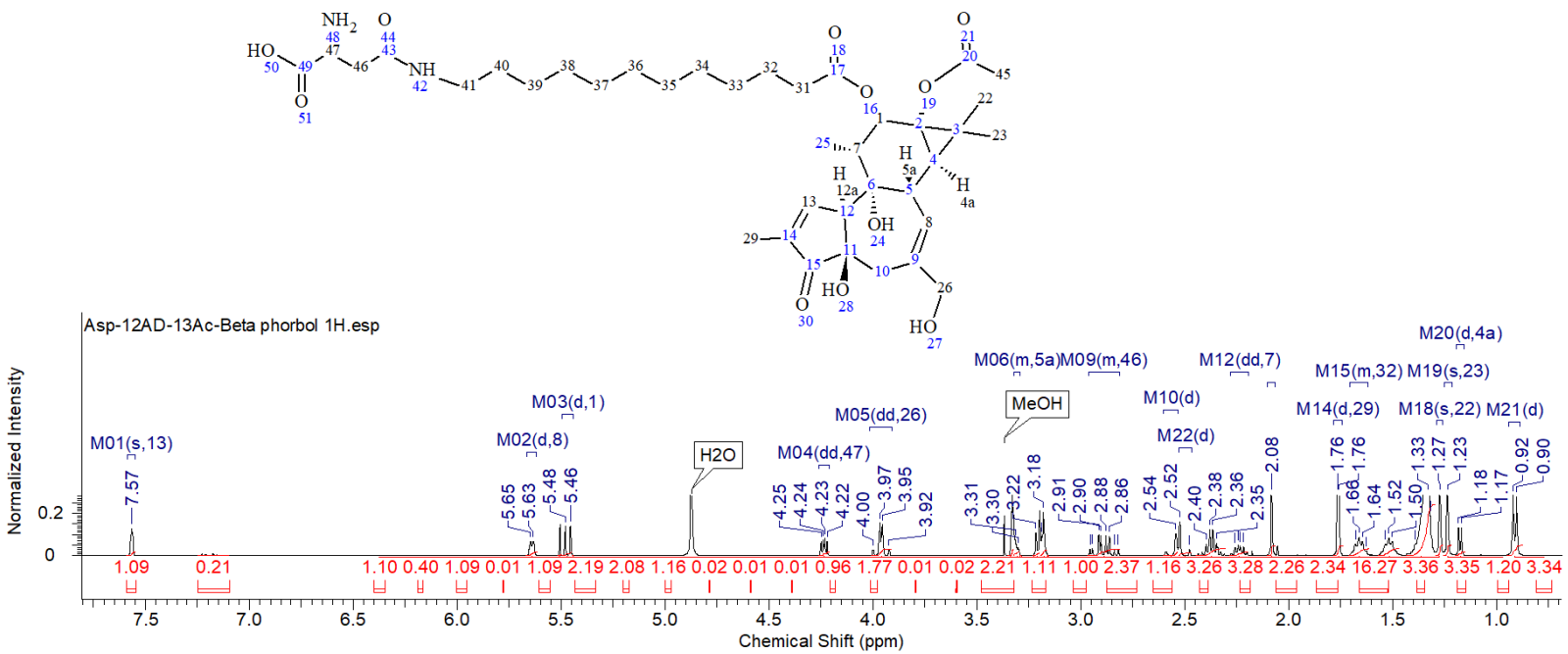

Fig.S7: Compound 3 ^{1}H NMR

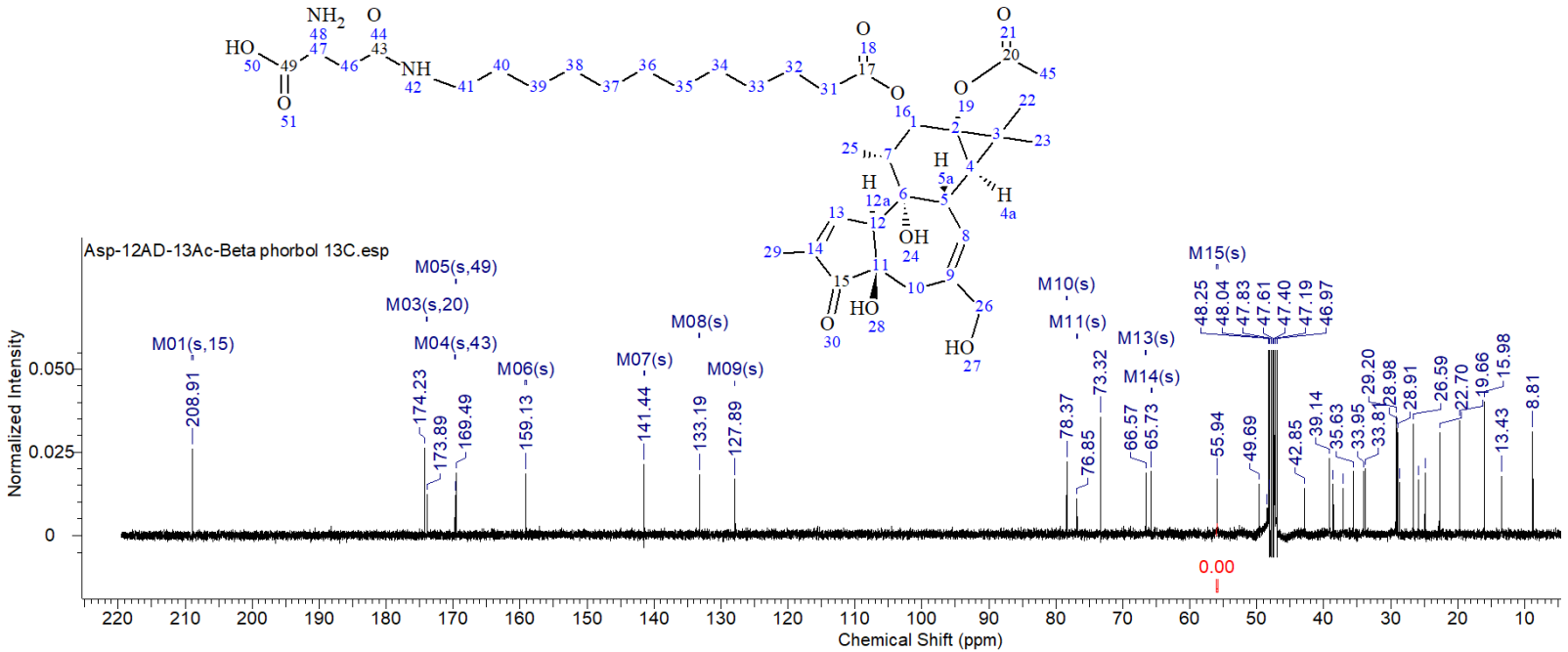

Fig.S8: Compound 3 ^{13}C NMR

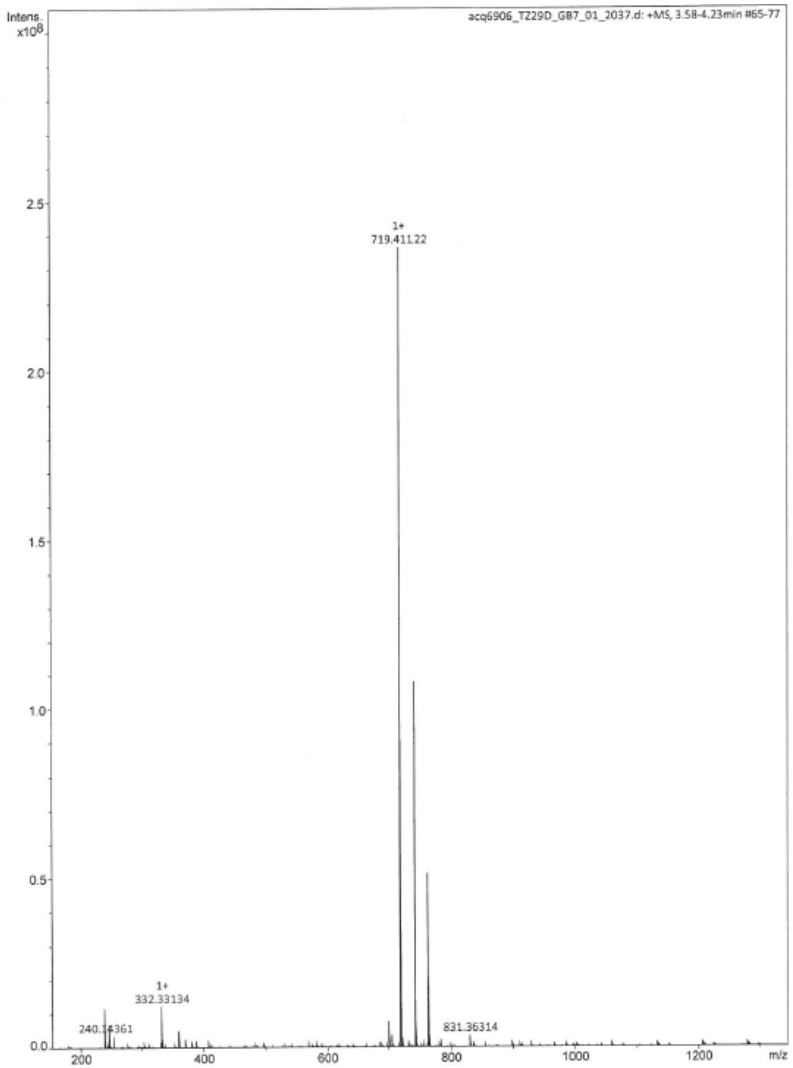

Fig.S9: Compound 3 HRMS

*S2.5.7. 12-O-(Leu-12-Aminododecanoyl)-13-O-Ac-β-phorbol (**2**)*

Fmoc-12-aminododecanoic loaded on 2-CTC resin (0.5 g, 0.48 mmol loading) was deprotected as described in the synthesis of **3**, then a solution of Boc-Leu-OH (170 mg; 0.71 mmol), PyBOP (372 mg; 0.71 mmol) and DIPEA (0.12 mL; 1.31 mmol) dissolved in DMF (5 mL) were added. The mixture was shaken for 4 h at 23 °C, and after a standard wash of the resin, cleavage was performed with a 20% solution of HFIP in DCM (3 × 6 mL; each time for 30 min). The eluate was concentrated to give *N*-Boc-Leu-12-aminododecanoate (90 mg; 88%) as a transparent thick amorphous solid. To a solution of Boc-Leu-12-aminododecanoic acid (0.090 g; 0.21 mmol) in DCM (3 mL) was added 13-*O*-Ac-20-*O*-TBDMS-4β-phorbol (**9**; 0.090 g; 0.17 mmol), DMAP (32 mg; 0.26 mmol) and EDC (0.050 g; 0.26 mmol) under stirring at 23 °C. After 15 h the reaction mixture was concentrated, and the residue purified on a silica column (heptane–EtOAc 1:1) to provide 12-*O*-(Boc-Leu-12-aminododecanoyl)-13-*O*-Ac-20-TBDMS-4β-phorbol (**13**) as white powder (140 mg; 94%).

A portion of the product **13** (74 mg; 0.09 mmol) was dissolved in DCM (3 mL), and then the Boc group was removed by addition of TFA (3 mL) and leaving the solution at 23 °C for 3 h. The reaction mixture was concentrated, and the residue fractionated by preparative HPLC with the gradient 30% → 100% B during 20 min to give **2** as a white powder (19.5 mg; 30%). Analytical UHPLC with gradient 20% → 80% B during 10 min: retention time 7.8 min; purity ≥99%. ^{1}H NMR (600 MHz, methanol-d_4) δ ppm 7.56 (dd, J = 2.1, 1.3 Hz, 1 H, H-1), 5.64 (d, J = 4.4 Hz, 1 H, H-7), 5.47 (d, J = 10.0 Hz, 1 H, H-12), 3.96 (dd, J = 12.8, 9.8 Hz, 2 H, H-20), 3.81 (t, J = 6.8 Hz, 1 H, α-H Leu), 3.31 (m, 1 H, H-8), 3.29 (t, J = 7.3 Hz, 1 H, H_a-12 12AD), 3.20 (t, J = 7.0 Hz, 1 H, H_b-12 12AD), 3.17 (m, 1 H, H-10), 2.58 - 2.48 2.57 (d, J = 19.1 Hz, 1 H, H_a-5), 2.50 (d, J = 19.1 Hz, 1 H, H_b-5), 2.42 - 2.32 (m, 2 H, H-2 12AD), 2.23 (m, 1 H, H-11), 2.08 (s, 3 H, H-2 Ac), 1.76 (dd, J = 2.8, 1.3 Hz, 3 H, H-19), 1.73 - 1.70 (m, 1 H, γ-H Leu), 1.70 - 1.67 (m, 2 H, β-H Leu), 1.67 - 1.63 (m, 2 H, H-11 12AD), 1.55 (quin, J = 7.2 Hz, 2 H, H-3 12AD), 1.40 - 1.32 (m, 14 H, H´-10 → H´-4), 1.27 (s, 3 H, H-16), 1.23 (s, 3 H, H-17), 1.17 (d, J = 5.4 Hz, 1 H, H-14), 1.03 - 1.00 (m, 6 H), 0.91 (d, J = 6.6 Hz, 3 H, H-18). ^{13}C NMR (150 MHz, methanol-d_4) δ ppm 208.87 (1 C, C-3), 174.19 (1 C, C´-1), 173.85 (1 C, C-1 Ac), 169.01 (1 C, C-1 Leu), 159.09 (1 C, C-1), 141.43 (1 C, C-6), 133.17 (1 C, C-2), 127.86 (1 C, C-7), 78.36 (1 C, C-9), 76.84 (1 C, C-12), 73.31 (1 C, C-4), 66.56 (1 C, C-20), 65.71 (1 C, C-13), 55.93 (1 C, C-10), 51.74 (1 C, C-2 Leu), 42.85 (1 C, C-11), 40.44 (1 C, C-3 Leu), 39.27 (1 C, C´-12), 38.59 (1 C, C-8), 37.06 (1 C, C-5), 35.63 (1 C, C-14), 33.93 (1 C, C´-2), 29.24-28.64 (7 C, C´-4 - C´-9 + C´-11), 26.57 (1 C, C´-10), 25.78 (1 C, C-15) 24.82 (1 C, C´-3), 24.12 (1C , C-4 Leu), 22.69 (1 C, C-17), 21.53 (1 C, C-5$_a$ Leu), 20.87 (1 C, C-5$_b$ Leu), 19.68 (1C, C-2 Ac), 15.96 (1 C, C-16), 13.42 (1 C, C-18), 8.80 (1 C, C-19). HRMS-ESI: $[C_{40}H_{64}N_2O_9+H]^+$ *m/z:* 717.4690. Found 717.4679.

Sample ID :
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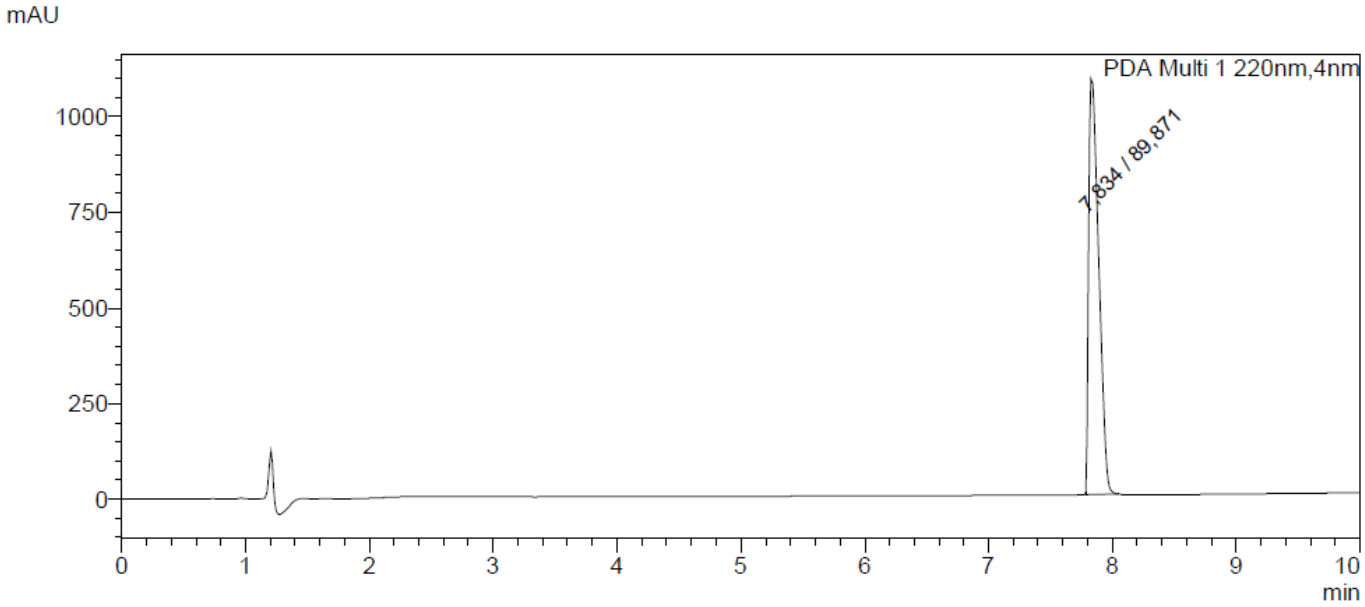
Method Filename : 20_80B_10min_purified2014.lcm
Batch Filename : 2017_05_23_1.lcb
Vial # : 1-63
Injection Volume : 10 uL
Date Acquired : 5/23/2017

Complete Chromatogram



PDA Ch1 220nm

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Fig. S10: Compound 2 analytical HPLC

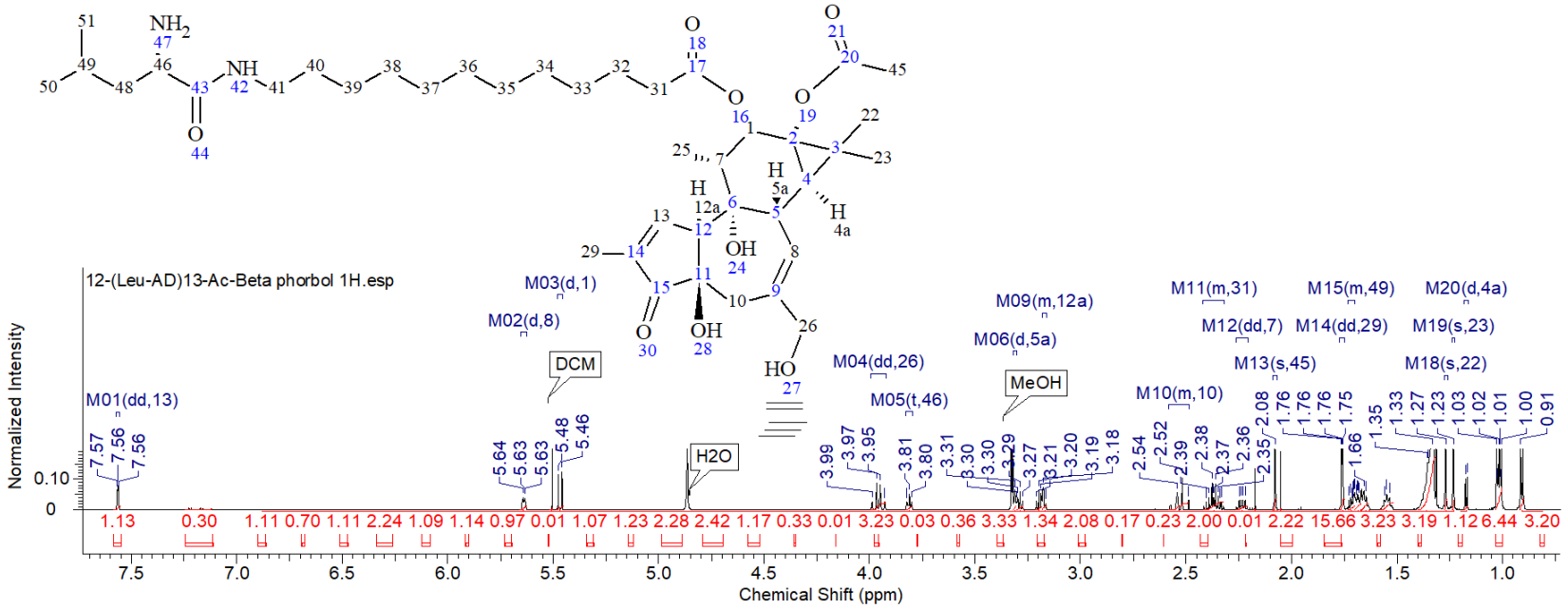

Fig.S11: Compound 2 ^{1}H NMR

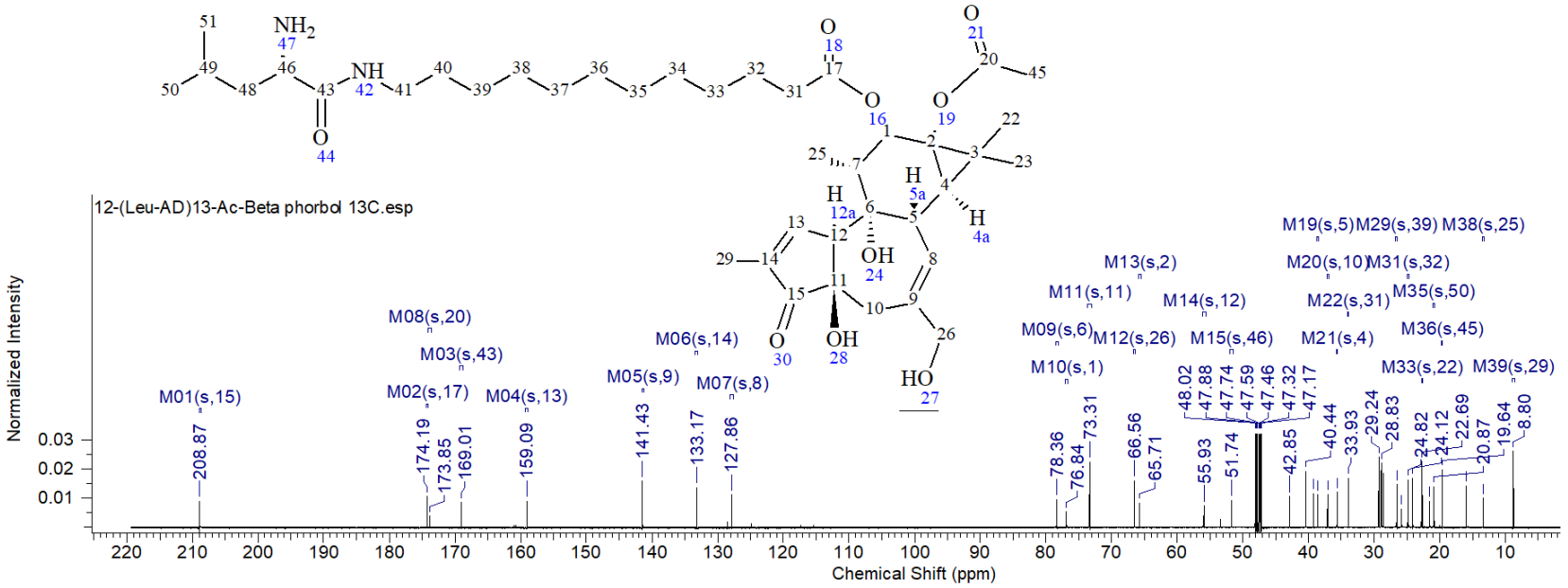

Fig.S12: Compound 2 ^{13}C NMR

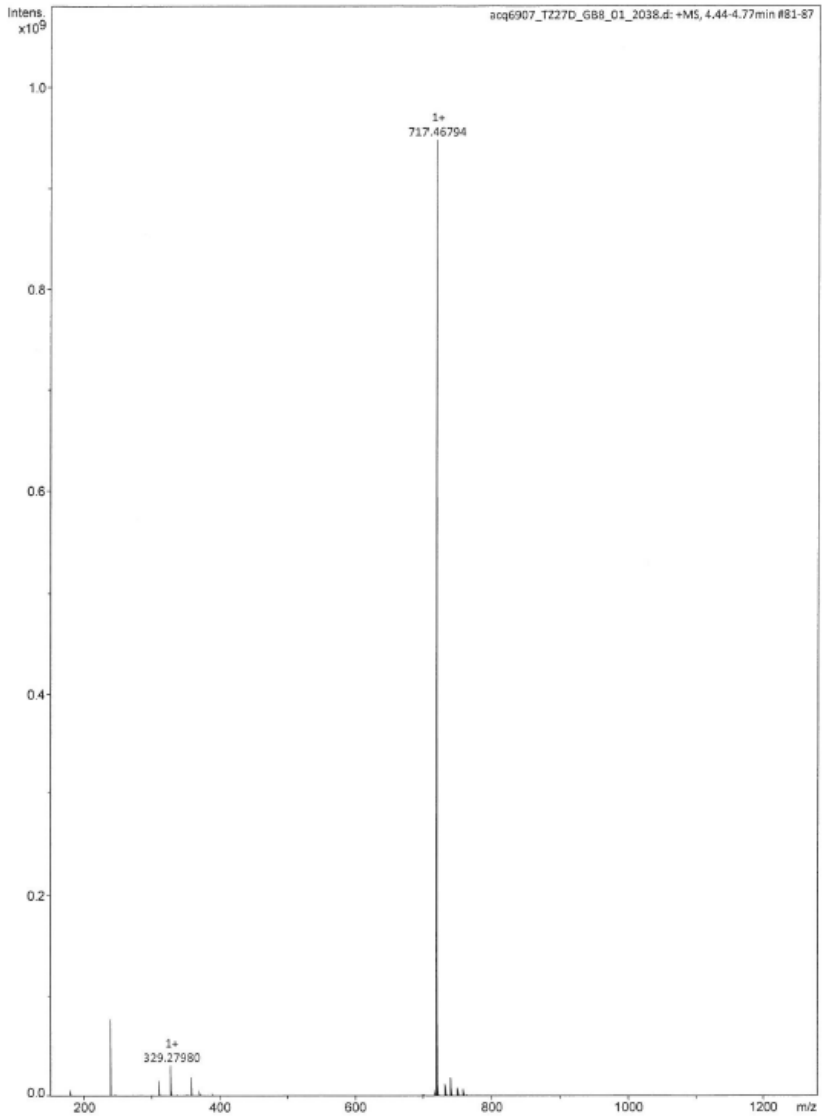

Fig.S13: Compound 2 HRMS

*S2.5.8. 12-O-(Morpholine-4-carbonyl-His-Ser-Ser-Lys-Leu-Gln-Leu-N-aminododecanoyl)-13-O-Ac-4β-phorbol (**5**)*

Synthesis of the peptide His-Ser-Ser-Lys-Leu-Gln-Leu was performed on a Liberty Blue microwave peptide synthesizer starting from preloaded Fmoc-Leu-(Cl-Trt)-resin (0.9 mmol/g; 110 mg; 0.1 mmol) swelled in DMF (5 mL). Calculated amounts of Fmoc protected amino acids in 0.2 M solution (added as volumes corresponding to 5 equivalents): Fmoc-His(Boc)-OH (0.38 g; 6 mL DMF), Fmoc-Ser(*t*Bu)-OH (0.43 g; 11 mL DMF), Fmoc-Lys(Boc)-OH (0.29 g; 6 mL DMF), Fmoc-Leu-OH (0.22 g; 6 mL DMF), Fmoc-Gln(Trt)-OH (0.37 g; 6 mL DMF). Conditions for chain elongation: single coupling at 45 °C during 15 min; Fmoc removal was performed with a 20% solution of piperidine in DMF at 45 °C for 30 s and 180 s.

The protected His-Ser-Ser-Lys-Leu-Gln-Leu-*O*-(Cl-Trt)-resin was placed in two reaction vessels for swelling. The resin was washed twice with DMF and DCM. A mixture of morpholine-4-carbonyl chloride–Et_3N–NMP (1:4:20; 8 mL) were added, and then the mixture was heated to 40 °C for 2 h. The resin was washed with DCM and the procedure was repeated for 1 h with fresh reagents, followed by washing with DMF, MeOH and DCM (each 3 × 8 mL for 3 min). The product was cleaved from the resin with a 20% solution of HFIP in DCM, and concentration of the drained cleavage mixture gave the protected peptide as an amorphous solid (220 mg). To a solution of the residue (134 mg) in DCM (1 mL) and THF (1 mL) was added PyBOP (57 mg; 0.11 mmol) and DIPEA (0.030 mL; 0.29 mmol). The mixture was stirred for 20 min, and then **11** (55 mg; 0.091 mmol) dissolved in DCM (1 mL) was added. The mixture was stirred under argon for 27 h at 23 °C. The resulting reaction mixture was concentrated, and the residue was dissolved in DCM (1.5 mL) and deprotected with TFA (1.5 mL; added four drops of H_2O) for 1 h at 23 °C. The cleavage mixture was concentrated, and the residue fractionated by preparative HPLC using the gradient 20 % → 70 % B over 20 min. The purity of the product was determined by analytical UHPLC (> 99%) by using the gradient 20% → 100 % B during 10 min (retention time: 6.2 min). Upon concentration the resulting residue was dissolved in 1,4-dioxane–H_2O and freeze-dried to provide prodrug **5** (14 mg; 10%) as a white powder. ^{1}H NMR (600 MHz, methanol-d_4) δ ppm 8.70 (s, 1 H, H-ε_1 His), 7.45 (s, 1 H, C-3), 7.27 (s, 1 H, H-δ_2 His), 5.52 (d, *J* = 4.4 Hz, 1 H, H-7), 5.35 (d, *J* = 10.0 Hz, 1 H, H-12), 4.48 (m, 1 H, α-H His), 4.39 - 4.34 (m, 2 H, 2 α-H Ser), , 4.21 (m, 1 H, α-H Lys), , 4.17 - 4.14 (m, 2 H, 2 α-H Leu), 4.12 (m, 1 H, α-H Gln), 3.87 - 3.80 (m, 2 H, 2 β H_a Ser), 3.85 - 3.82 (m, 2H, H-20), 3.74 - 3.69 (m, 2 H, 2 β H_b Ser), 3.57 - 3.51 (t, *J* = 4.4 Hz, 4 H, H-2 and H-6 morpholine), 3.31 - 3.27 (t, *J* = 5.1 Hz, 4 H, H-3 and H-5 morpholine), 3.20 (m, 1 H, β –H_a His), 3.19 (m, 1 H, H-8), 3.11 (m, 1 H, β –H_b His), 3.06 (m, 2 H, H-12 12AD), 3.03 (m, 1 H, H-10), 2.83 (t, *J* = 7.5 Hz, 2 H, ε-H Lys), 2.43 (d, *J* = 19.1 Hz, 1 H, H_a-5), 2.40 (d, *J* = 19.1 Hz, 1 H, H_b-5), 2.30 - 2.25 (m, 2 H, γ-H Gln), 2.25-2.19 (m, 2 H, H-2 12AD), 2.12 (m, 1 H, H-11), 2.05 - 1.97 (m, 2 H, β-H Gln), 1.96 (s, 3 H,

H-2 Ac), 1.79 (m, 1 H, β-H_a Lys), 1.69 (m, 1 H, β-H_b Lys), 1.64 (dd, *J* = 2.8, 1.3 Hz, 3 H, H-19), 1.62 - 1.59 (m, 2 H, 2 γ-H Leu), 1.58 (m, 2 H, δ-H Lys), 1.57 – 1.52 (m, 4 H, 2 β-H Leu), 1.50 (m, 2 H, H-3 12AD), 1.42 (m, 2 H, H-11 12AD), 1.40 (m, 2 H, γ-H Lys), 1.29 - 1.20 (m, 14 H, H-10 → H-4 12AD), 1.15 (s, 3 H, H-16), 1.12 (s, 3H, H-17), 1.06 (d, *J* = 5.4 Hz, 1 H, H-14) 0.85 (m, 6 H, 2 δ-H_a Leu), 0.82-0.80 (m, 6 H, 2 δ-H_b Leu), 0.79 (d, *J* = 6.6 Hz, 3 H, H-18).^{13}C NMR (150 MHz, methanol-d_4) *δ* ppm 208.94 (1 C, C-3), 176.41 (1 C, C-5 Gln**),** 174.47 (1 C, C´-1), 174.22 (1 C, C-1 Ac)**,** 173.94 (1 C, C-1 Leu_5), 173.90 (1 C, C-1 Leu_7), 173.48 (1 C, C-1 Lys), 173.15 (1 C, C-1 His), 172.39 (1 C, C-1 Gln), 172.21 (1 C, C-1 Ser_2), 172.05 (1 C, C-1 Ser_3), 159.10 (1 C, C-1), 157.97 (1 C, morpholine carbonyl), 141.45 (1 C, C-6), 133.60 (1 C, C-$ε_1$ His)**,** 133.19 (1 C, C-2), 129.94 (1 C, H-γ His), 127.89 (1 C, C-7), 117.12 (1 C, C-$δ_2$ His), 78.37 (1 C, C-9), 76.84 (1 C, C-12), 73.32 (1 C, C-4), 66.57 (1 C, C-20), 66.12 (2 C, C-2 and C-6 morpholine), 66.72 (1 C, C-13), 61.46 (1 C, C-3 Ser_3), 61.32 (1 C, C-3 Ser_2), 55.94 (1 C, C-10) 55.63 (2 C, C-2 Ser), 54.35 (1 C, C-2 Gln), 54.14 (1 C, C-2 His), 53.58 (2 C, C-2 Leu), 52.12 (1 C, C-2 Lys), 43.93 (2 C, C-3 and C-5 morpholine), 42.85 (1 C, C-11), 39.61 (2 C, C-3 Leu), 39.11 (2 C, C´-12 and C-6 Lys), 38.60 (1 C, C-8), 37.07 (1 C, C-5), 35.63 (1 C, C-14), 33.95 (1 C, C´-2), 31.42 (1 C, C-4 Gln), 30.10 (1 C, C-3 Lys), 29.25-28.66 (7 C, C´-4 - C´-9 + C´-11), 27.00 (1 C, C-3 Gln)**,** 26.73 (1 C, C-3 His), 26.56 (1 C, C-5 Lys), 26.50 (1 C, C´-10), 25.76 (1 C, C-15), 24.84 (1 C, C´-3), 24.48 (2 C, 2 C-4 Leu), 24.60 (1 C, C-5 Leu_5), 24.51 (1 C, C-5 Leu_7), 22.81 (1 C, C-17), 22.71 (1 C, C-4 Lys), 22.23 + 22.00 (2 C, 2 C-6 Leu), 20.47 + 20.30 (2 C, 2 C-6 Leu), 19.67 (1 C, C-2 Ac), 15.98 (1 C, C-16), 13.42 (1 C, C-18), 8.81 (1 C, C-19). HRMS-ESI: $[C_{74}H_{119}N_{13}O_{20}+ 2H]^{2+}$ *m/z:* 755.9425. Found 755.9406.

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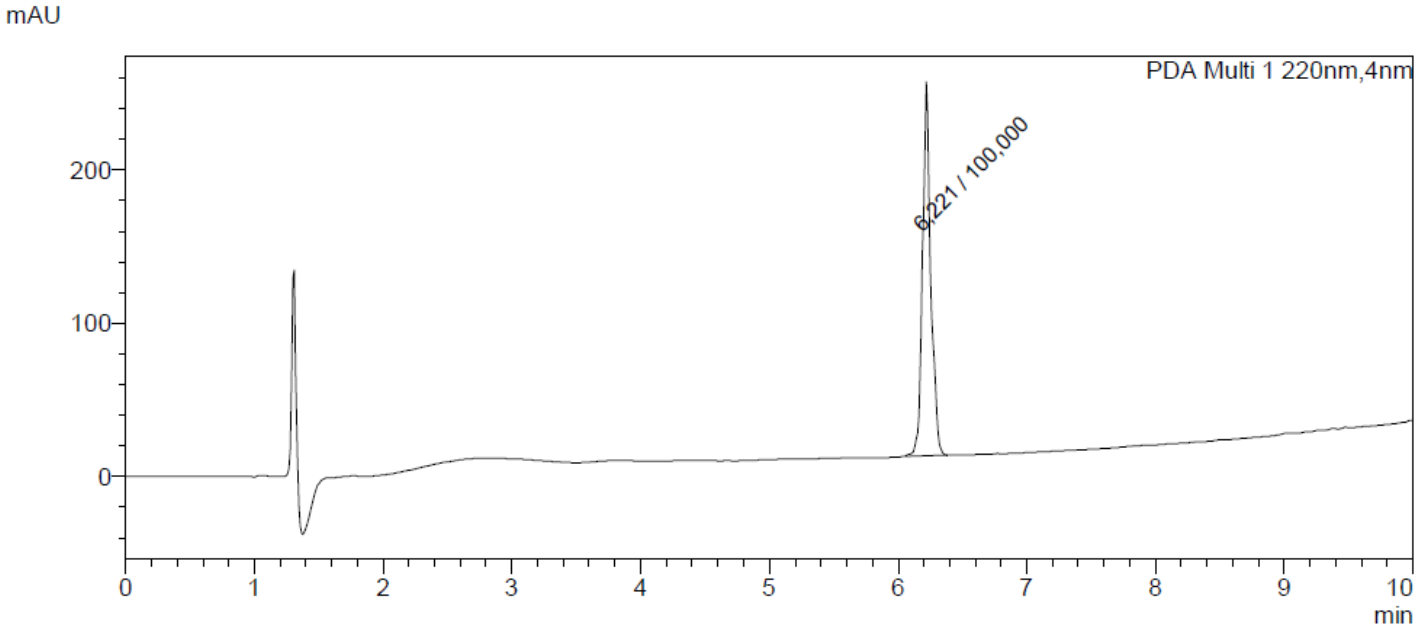
Date Acquired : 12/20/2017

Complete Chromatogram



PDA Ch1 220nm

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Fig.S14: Compound 5 HPLC

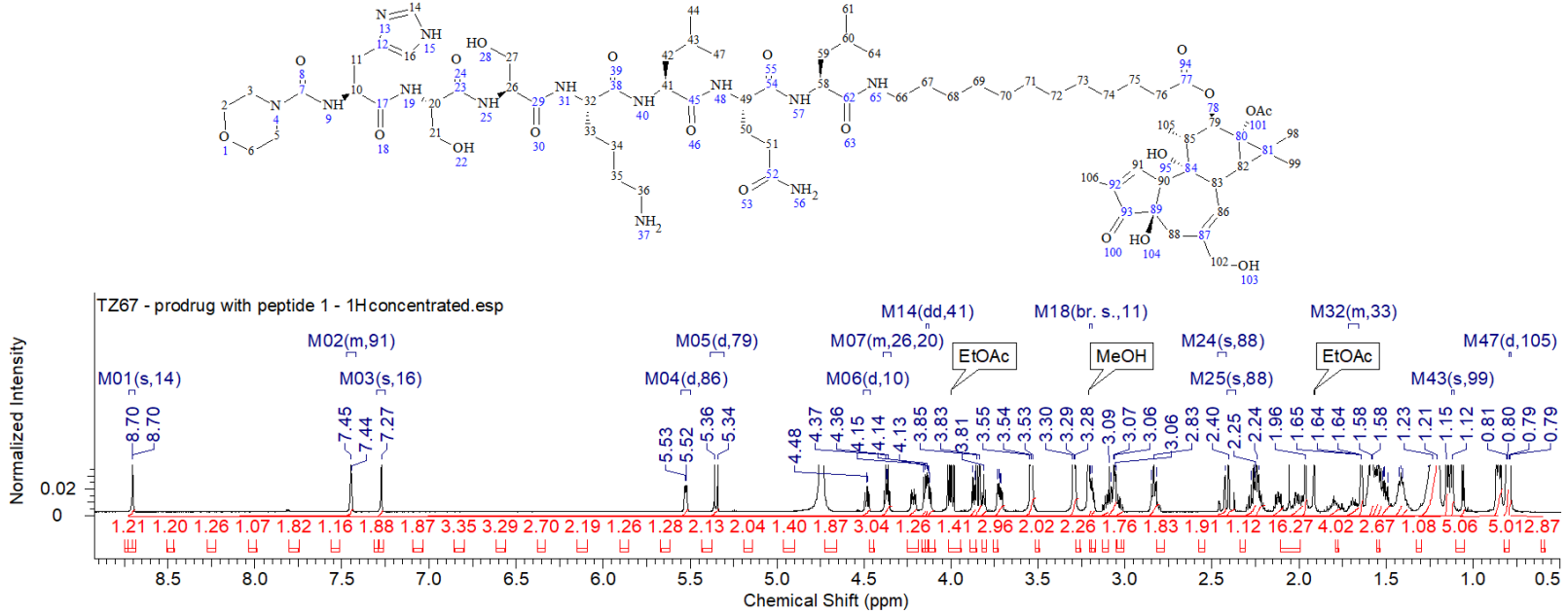

Fig.S15: Compound 5 ^{1}H NMR

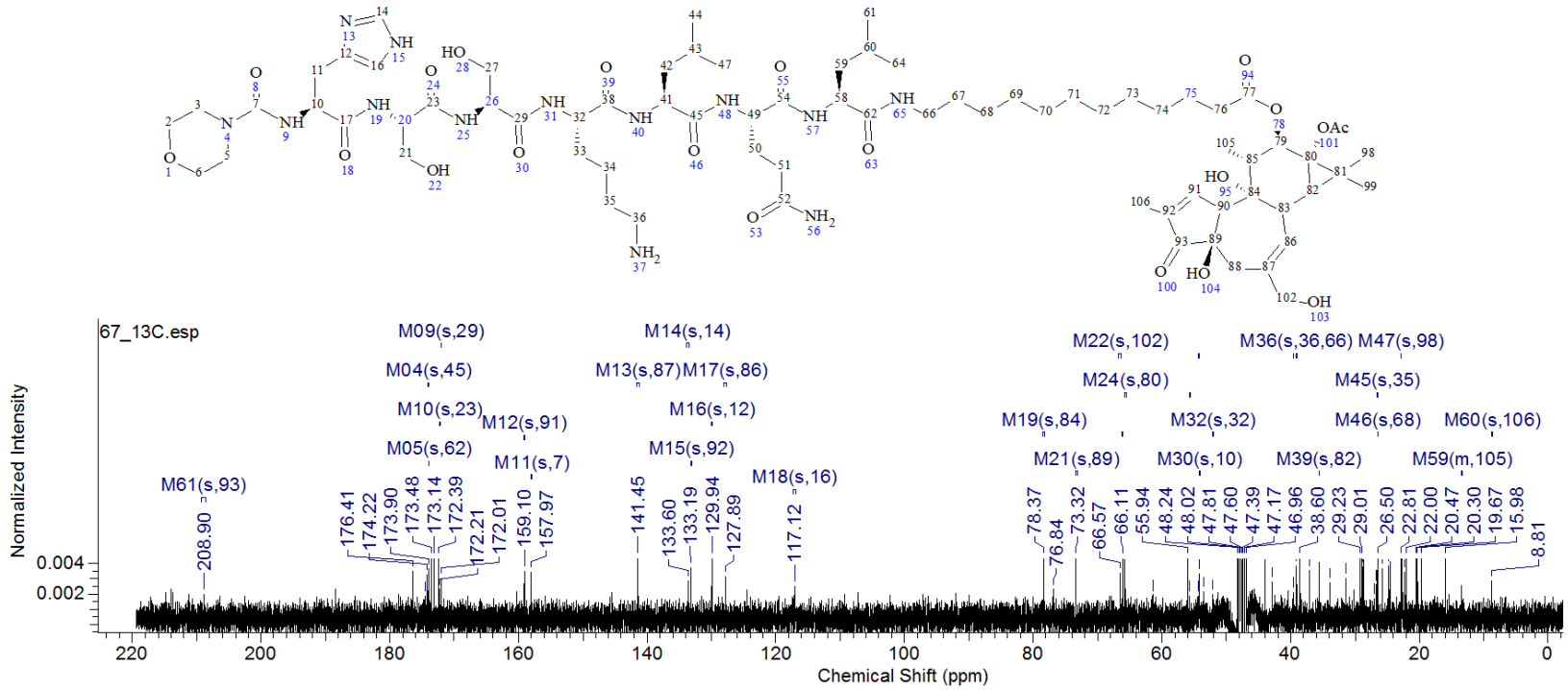

Fig.S16: Compound 5 ^{13}C NMR

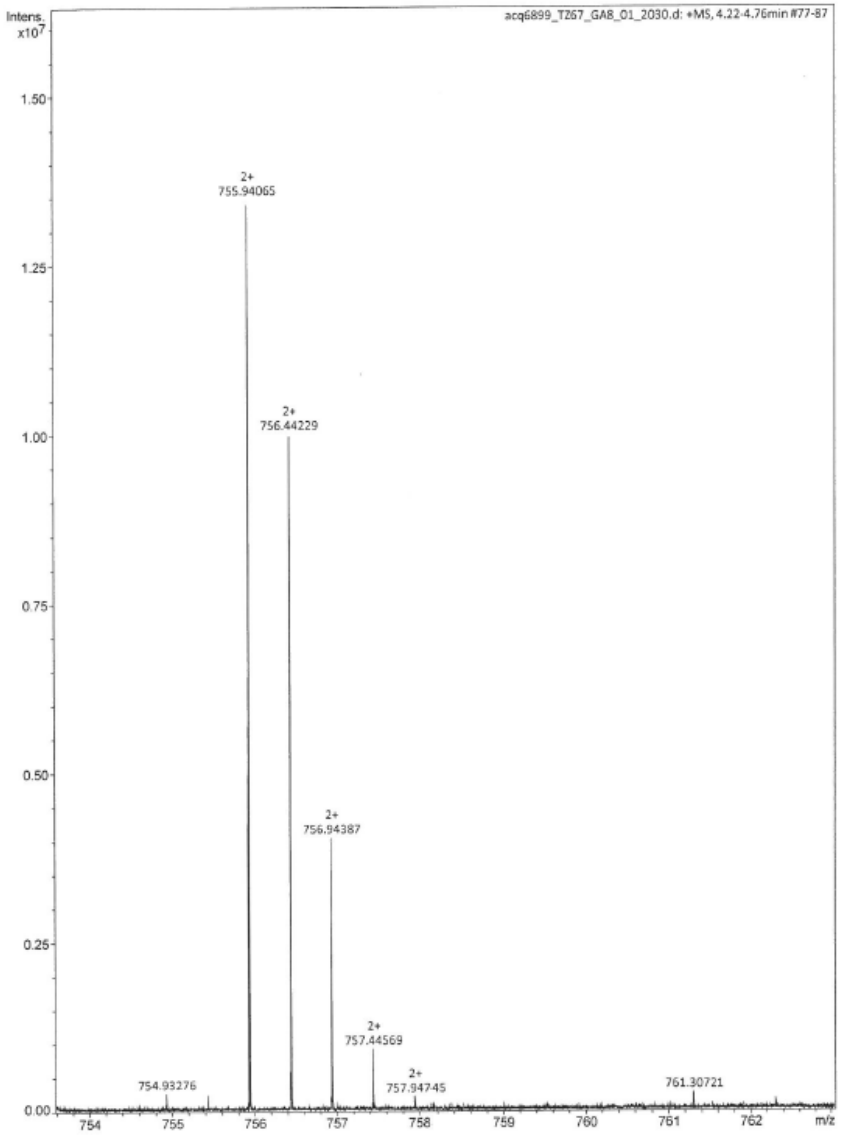

Fig.S17: Compound 5 HRMS

*S2.5.9. 12-O-(Ac-Gly-Lys-Ala-Phe-Arg-Arg-Leu-N-aminododecanoyl)-13-O-Ac-4β-phorbol (**4**)*

First, the C-terminal residue was loaded onto a 2-CTC resin (1.6 mmol/g; 1.0 g) in DCM (4 mL) by addition of Fmoc-Leu-OH (564 mg; 1.60 mmol) dissolved in DCM (4 mL) with DIPEA (2.7 mL; 16 mmol) added. The mixture was shaken for 3.5 h at 23 °C, and the resin was washed with DCM, free linker groups were capped by treatment with DIPEA–MeOH–DCM 5:15:80 (8 mL; 2 × 5 min), and subsequently the resin was washed with DMF, MeOH and DCM (each with 3 × 10 mL for 3 min). Test cleavage with 20% HFIP-DCM (2 mL) of a small amount of dry loaded resin was followed by thorough evaporation and weighing the amount of cleaved Fmoc-Leu-OH showed a loading of approx. 0.9 mmol/g.

The peptide sequence Ac-Gly-Lys-Ala-Phe-Arg-Arg-Leu-OH was assembled by using a Liberty Blue microwave peptide synthesizer starting from Fmoc-Leu-*O*-(2-Cl-Trt)-resin (0.9 mmol/g; 110 mg; 0.1 mmol) swelled in DMF (5 mL) as a starting material. Calculated amounts of Fmoc protected amino acids in 0.2 M solution (added as volumes corresponding to 5 equivalents): Fmoc-Gly-OH (0.33 g; 11 mL DMF), Fmoc-Lys(Boc)-OH (0.52 g; 11 mL DMF), Fmoc-Ala-OH (0.35 g; 11 mL DMF), Fmoc-Phe-OH (0.43 g; 11 mL DMF), Fmoc-Arg(Pbf)-OH (2.08 g; 32 mL DMF). Conditions for chain elongation: double coupling at 45 °C during 15 min, while triple coupling at 45 °C during 15 min was needed for Arg; Fmoc removal was performed with a 20% solution of piperidine in DMF at 45 °C for 30 s and 180 s.

The obtained side-chain protected Gly-Lys-Ala-Phe-Arg-Arg-Leu-*O*-(Cl-Trt)-resin was washed twice with both DMF and DCM followed by acetylation with Ac_2O–DIPEA–NMP (1:2:3, 6 mL; 2 × 5 min). The resin-bound protected peptide was washed with DMF, MeOH and DCM (each 3 × 8 mL for 3 min), and cleaved from the resin with a 20% solution of HFIP in DCM. The drained solution was concentrated to give the protected peptide as an amorphous solid. To a solution of the residue (158 mg; 0.11 mmol) in DCM (1 mL) and THF (1.5 mL) was added PyBOP (66 mg; 0.13 mmol) and DIPEA (27 mg; 0.20 mmol) in DCM (0.5 mL). The mixture was stirred for 20 min to pre-activate the carboxylic acid, and then a solution of compound **8** (64 mg; 0.10 mmol) in DCM (1.5 mL) was added. The mixture was stirred under argon for 14 h at 23 °C, and the resulting reaction mixture was concentrated *in vacuo*. The obtained residue was deprotected with TFA (3 mL added four drops of H_2O) for 18 h at 23 °C. After this deprotection a by-product with one Pbf-protected Arg residue was still present as judged by MALDI. After a second deprotection with TFA (3 mL added four drops of water) for 3 h. the reaction mixture was concentrated, and the residue subjected to purification by preparative HPLC using the gradient 20% → 100 % B over 20 min. The purity of the product was estimated by analytical UHPLC to be >99% by using the gradient 20% → 100 % B during 10 min (retention time: 5.3 min). Upon concentration the product was dissolved in 1,4-dioxane–H_2O and freeze-dried to provide prodrug **4** (18 mg; 12%) as a white solid. ^{1}H NMR (600 MHz,

methanol-d_4) δ ppm 7.44 (s, 1 H, C-3), 7.21 – 7.07 (s, 5 H, H-Phe aromatics), 5.52 (d, J = 4.4 Hz, 1 H, H-7), 5.35 (d, J = 10.0 Hz, 1 H, H-12), 4.34 (m, 1 H, α-H Phe), 4.23 (m, 1 H, α-H Lys), , 4.18 (m, 1 H, α-H Arg), , 4.13 (m, 1 H, α-H Arg), 4.08 (m, 1 H, α-H Leu), 4.04 (m, 1 H, α-H Ala), 3.89 – 3.81 (m, 2H, H-20), 3.84 - 3.71 (m, 2 H, α-H Gly), 3.18 (m, 1 H, H-8), 3.14 - 3.06 (m, 6 H, 2 δ-H Arg and H-12 12AD), 3.05 (m, 1H, H-10), 3.03 – 2.91 (m, 1 H, β-H Phe) 2.84 (t, J = 7.5 Hz, 2 H, ε-H Lys), 2.45 (d, J = 19.1 Hz, 1 H, H_a-5), 2.41 (d, J = 19.1 Hz, 1 H, H_b-5), 2.30-2.20 (m, 2 H, H-2 12AD), 2.12 (m, 1 H, H-11), 1.96 (s, 3 H, H-2 phorbol Ac), 1.88 (s, 3 H, H-2 capped Ac), 1.84 – 1.72 (m, 4 H, β-H Arg), 1.71 – 1.66 (m, 2H, β-H Lys), 1.64 (dd, J = 2.8, 1.3 Hz, 3 H, H-19), 1.61 (m, 1 H, γ-H Leu), 1.60 – 1.57 (m, 4 H, γ-H Arg), 1.56 (m, 2 H, H-11 12AD), 1.54 (m, 2 H, β-H Leu), 1.51 – 1.45 (m, 2 H, δ-H Lys), 1.45 – 1.40 (m, 2 H, H-3 12AD), 1.39 – 1.33 (m, 2 H, γ-H Lys), 1.29 - 1.20 (m, 14 H, H-10 $\rightarrow$ H-4 12AD), 1.16 (s, 3 H, H-16), 1.11 (s, 3H, H-17), 1.06 (d, J = 5.4 Hz, 1 H, H-14), 0.84 (d, J = 6.6 Hz, 3 H, H-18), 0.82-0.77 (m, 6 H, 2 δ-H Leu). ^{13}C NMR (150 MHz, methanol-d_4) δ ppm 208.90 (1 C, C-3), 174.55 (1 C, C-1 Leu), 174.23 (1 C, C´-1), 173.89 (1 C, C-1 phorbol Ac), 173.64 (1 C, C-1 Ala), 173.19 (2 C, C-1 Arg), 172.98 (1 C, C-1 Phe), 172.87 (1 C, C-1 capped Ac), 172.40 (1 C, C-1 Lys), 171.66 (1 C, C-1 Gly), 159.11 (1 C, C-1), 157.27 + 157.25 (2 C, C-6 Arg), 141.44 (1 C, C-6), 136.90 (1 C, C-4 Phe), 133.19 (1 C, C-2), 128.82 (2 C, C-5 + C-9 Phe), 128.19 (2 C, C-6 + C-8 Phe), 127.89 (1 C, C-7), 126.53 (1 C, C-7 Phe), 78.37 (1 C, C-9), 76.85 (1 C, C-12), 73.32 (1 C, C-4), 66.57 (1 C, C-20), 65.73 (1 C, C-13), 55.94 (1 C, C-10), 55.9 (1 C, C-2 Phe), 54.75 (1 C, C-2 Leu), 53.79 (1 C, C-2 Arg), 53.41 (C, C-2 Arg), 52.15 (1 C, C-2 Lys), 50.62 (1 C, C-2 Ala), 42.93 (1 C, C-2 Gly), 42.85 (1 C, C-11), 40.68 (1 C, C-3 Leu), 40.55 (2 C, C-5 Arg), 39.07 (2 C, C-12 12AD and C-6 Lys), 38.60 (1 C, C-8), 37.07 (1 C, C-5), 36.53 (1 C, C-Phe), 35.63 (1 C, C-14), 33.95 (1 C, C´-2), 30.33 (1 C, C-3 Lys), 29.29-28.66 (7 C, C´-4 - C´-9 + C´-11), 27.97 (2 C, C-3 Arg), 26.75 (2 C, C-4 Arg), 26.51 (1 C, C´-10), 25.79 (1 C, C-15), 24.97 (1 C, C-5 Lys), 24.84 (1 C, C-3 12AD), 24.48 (2 C, 2 C-4 Leu), 22.82 (1 C, C-17), 22.53 (1 C, C-4 Lys), 22.16 + 22.40 (2 C, 2 C-6 Leu), 21.13 (1 C, C-2 capped Ac), 19.67 (1 C, C-2 phorbol Ac), 15.98 (1 C, C-16), 13.43 (1 C, C-18), 8.81 (1 C, C-19). HRMS-ESI $C_{74}H_{119}N_{15}O_{16}$ + 2H]$^{2+}$ *m/z:* 737.9550. Found 737.9546.

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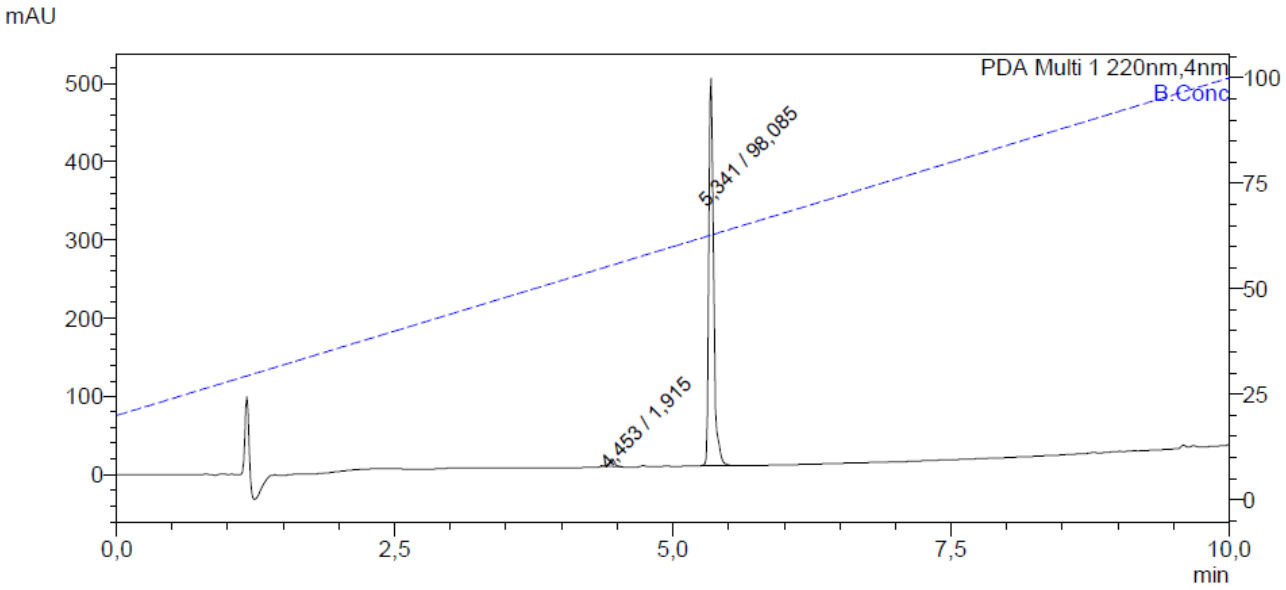
Date Acquired : 9/10/2017

Complete Chromatogram



PDA Ch1 220nm

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Fig.S18: Compound 4 HPLC

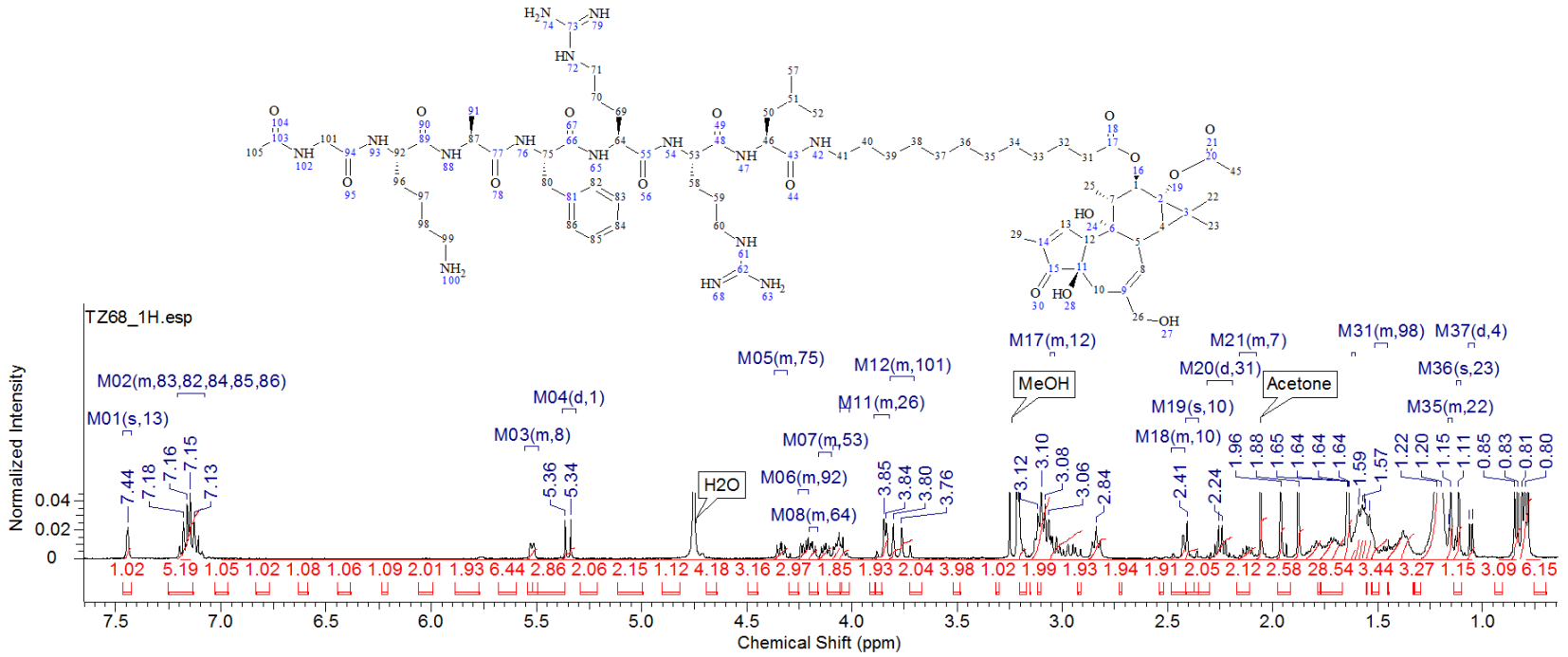

Fig.S19: Compound 4 ^{1}H NMR

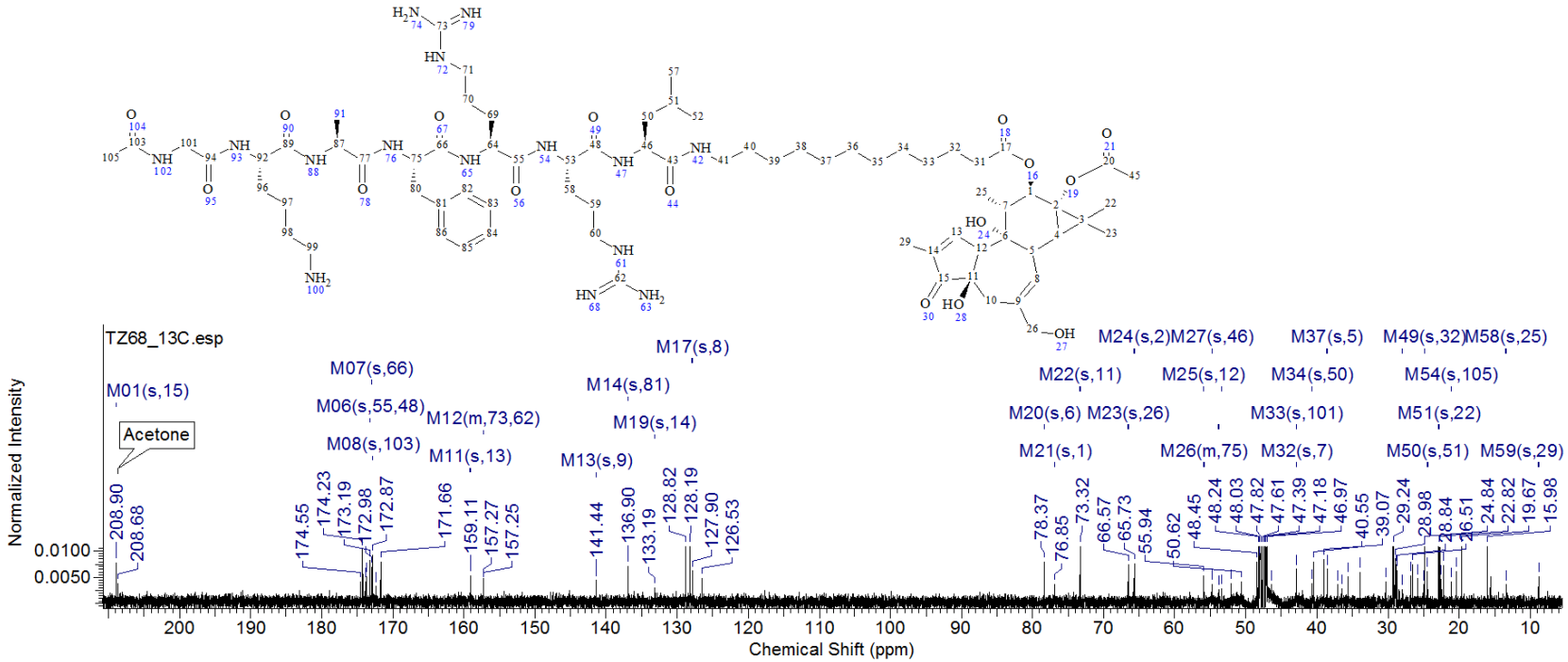

Fig.S20: Compound 4 ^{13}C NMR

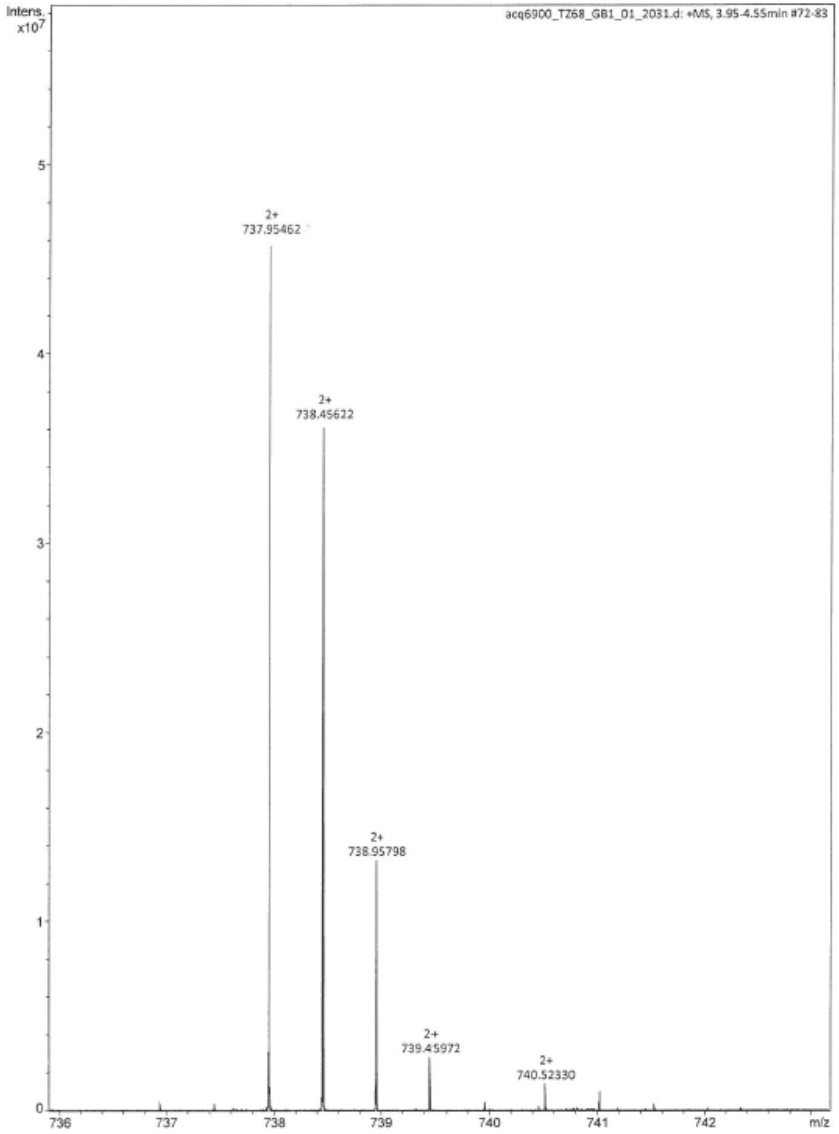

Fig.S21: Compound 4 HRMS

*S2.5.10. 12-O-((γ-Glu-)$_4$-β-Asp-N-Aminododecanoyl)-13-O-Ac-4β-phorbol (**6**)*

2-CTC resin (1.6 mmol/g; 3.125 g; 5 mmol) was swelled in DCM (8 mL) in a glass filter vessel (300 mL) for manual SPPS (Peptides International, Louisville, USA). After draining a solution of Fmoc-Glu-O*t*Bu (425 mg; 1.0 mmol) and DIPEA (2.1 mL; 12 mmol) in DCM (15 mL) was added to the resin and the mixture was shaken for 3 h at 23 °C. The resin was drained, washed with DCM, capped with DIPEA–MeOH–DCM 5:15:80 (8 mL; 2 × 5 min), and washed with DMF, MeOH and DCM (each 3 times for 3 min). The reaction mixture was transferred to glass flask, concentrated in vacuo and residual solvent in the resin was removed on a freeze-dryer. Test cleavage of a dry sample of the loaded resin showed a loading of ca. 0.3 mmol/g.
A large syringe (with a polypropylene filter bottom) was used as reaction vessel for assembly of the peptide on the resin preloaded with Fmoc-Glu-O*t*Bu (0.30 mmol/g; 1.50 g). Three sequential coupling cycles with addition of a solution of Fmoc-Glu-O*t*Bu (390 mg; 0.95 mmol) pre-activated (10 min) with PyBOP (711 mg; 1.37 mmol) and DIPEA (0.475 mL; 2.72 mmol) in DMF (3 mL). Each coupling was followed by Fmoc removal with a 20% solution of piperidine in DMF (8 mL; 2 × 20 min), washing of the resin with DMF, MeOH and DCM (each 3 times for 3 min), addition of DMF (3 mL) and DCM (2 mL). Each coupling was performed under shaking for 5 h at 23 °C, and then the resin was washed with DMF, MeOH and DCM (each 3 times for 3 min). Boc-Asp(All)-OH (0.350 g; 1.28 mmol) was coupled by using the same conditions. On-resin allyl deprotection was performed by adding DCM (5 mL) followed by a solution of $Me_2N{\cdot}BH_3$ in DCM (2 mL) and a 0.02 M solution of $Pd(PPh_3)_4$ (185 mg) in DCM (8 mL). The mixture was shaken for 6 h under argon at 23 °C, after which the resin was washed with DMF, MeOH and DCM (each 3 times for 3 min).
DCM (4 mL) was added to a part of the resin-bound peptide (1.5 g) and a solution of PyBOP (680 mg; 1.2 mmol) and DIPEA (0.47 mL; 2.7 mmol) in DCM (4 mL) was added to pre-activate the carboxylic acid for 20 min. A solution of **8** (340 mg; 0.56 mmol) in DCM (4 mL) was added, and the mixture was shaken for 14 h at 23 °C. Finally, the resin was drained and washed with DMF, MeOH and DCM (each 3 times for 3 min), and then the conjugate was cleaved from the resin with simultaneous removal of side-chain protecting groups by treatment with TFA (5 mL with 5 drops of H_2O added) for 2 h at 23 °C. Concentration of the reaction mixture afforded a crude product, which was purified by preparative HPLC using a gradient of 20% → 80% B (20 min) to give prodrug **6**, which by analytical UHPLC had a purity of >98% when using the same gradient 20% → 60% B (10 min; retention time 7.4 min). This fraction was evaporated to give a residue, which was dissolved in 1,4-dioxane–H_2O and freeze-dried to give prodrug **6** (55 mg; 12%) as a white solid. ^{1}H NMR (600 MHz, methanol-d_4) δ ppm 7.45 (s, 1 H, C-3), 5.52 (d, J = 4.4 Hz, 1 H, H-7), 5.35 (d, J = 10.0 Hz, 1 H, H-12), 4.38 (m, 1 H, α-H Glu$_4$), 4.36 - 4.31 (m, 3 H, α-H Glu$_{1-3}$), 4.20 (m, 1 H, α-H Asp), 3.84 (dd, J = 12.8, 9.8 Hz, 2 H, H-20), 3.20 (m, 1 H, H-

8), 3.13 - 3.07 (m, 2 H, H´-12), 3.06 (m, 1 H, H-10), 2.87 (dd, *J* = 4.4, 16.5 Hz, 1 H, H_a-β Asp), 2.71 (dd, *J* = 9.0, 16.7 Hz, 1 H, H_b-β Asp), 2.45 (d, *J* = 19.1 Hz, 1 H, H_a-5), 2.41 (d, *J* = 19.1 Hz, 1 H, H_b-5), 2.36 - 2.27 (m, 8 H, γ-H Glu), 2.26 (m, 2 H, H-2 12AD), 2.24 - 2.10 (m, 4 H, β-H Glu_1 + Glu_4), 2.08 (m, 1 H, H-11), 1.96 (s, 3 H, H-2 Ac), 1.93 - 1.80 (m, 4 H, β-H Glu_2 + Glu_3), 1.64 (dd, *J* = 2.8, 1.3 Hz, 3 H, H-19), 1.54 (quin, *J* = 7.2 Hz, 2 H, H´-3), 1.40 (quin, *J* = 6.8 Hz, 2 H, H´-11), 1.28 - 1.19 (m, 14 H, H´-10 → H´-4), 1.16 (s, 3 H, H-16), 1.12 (s, 3 H, H-17), 1.06 (d, *J* = 5.4 Hz, 1 H, H-14), 0.79 (d, *J* = 6.6 Hz, 3 H, H-18). ^{13}C NMR (150 MHz, methanol-d_4) *δ* ppm 208.93 (1 C, C-3), 174.99 (1 C, C-5 Glu_1) 174.25 (1 C, C´-1), 173.91 (1 C, C-1 Ac), 173.61 (4 C, C-1 Glu_{1-4}), 173.42 (1 C, C-5 Glu_2), 172.75 (2 C, C-5 Glu_{3-4}), 169.20 (1 C, C-1 Asp), 168.26 (1 C, C-5 Asp), 159.16 (1 C, C-1), 141.44 (1 C, C-6), 133.19 (1 C, C-2), 127.89 (1 C, C-7), 78.38 (1 C, C-9) 76.86 (1 C, C-12), 73.32 (1 C, C-4), 66.57 (1 C, C-20), 65.73 (1 C, C-13), 55.93 (1 C, C-10), 52.00 (1 C, C-2 Glu_1), 51.78 (1 C, C-2 Glu_4), 51.68 (2 C, C-2 Glu_{2+3}), 50.09 (1 C, C-2 Asp) 42.86 (1 C, C-11), 39.28 (1 C, C´-12), 38.61 (1 C, C-8), 37.07 (1 C, C-5), 35.63 (1 C, C-14), 35.12 (1 C, C-3 Asp), 33.94 (1 C, C´-2), 31.55 – 31.50 (1 C, C-4 Glu_{2-4}), 29.85 (1 C, C-4 Glu_1), 28.60 - 29.29 (7 C, 7 C, C´-4 - C´-9 + C´-11), 27.06 (1 C, C-3 Glu_1), 26.90 – 26.85 (2 C, C-3 Glu_{2+3}), 26.64 (1 C, C´-10), 26.46 (1 C, C-3 Glu_2), 25.79 (1 C, C-15), 24.83 (1 C, C´-3) 22.70 (1 C, C-17), 19.67 (1 C, C-2 Ac), 15.97 (1 C, C-16), 13.43 (1 C, C-18), 8.81 (1 C, C-19). HRMS-ESI: $[C_{58}H_{86}N_6O_{23}+H]^+$ *m/z:* 1235.5822 Found 1235.5818

Sample ID :
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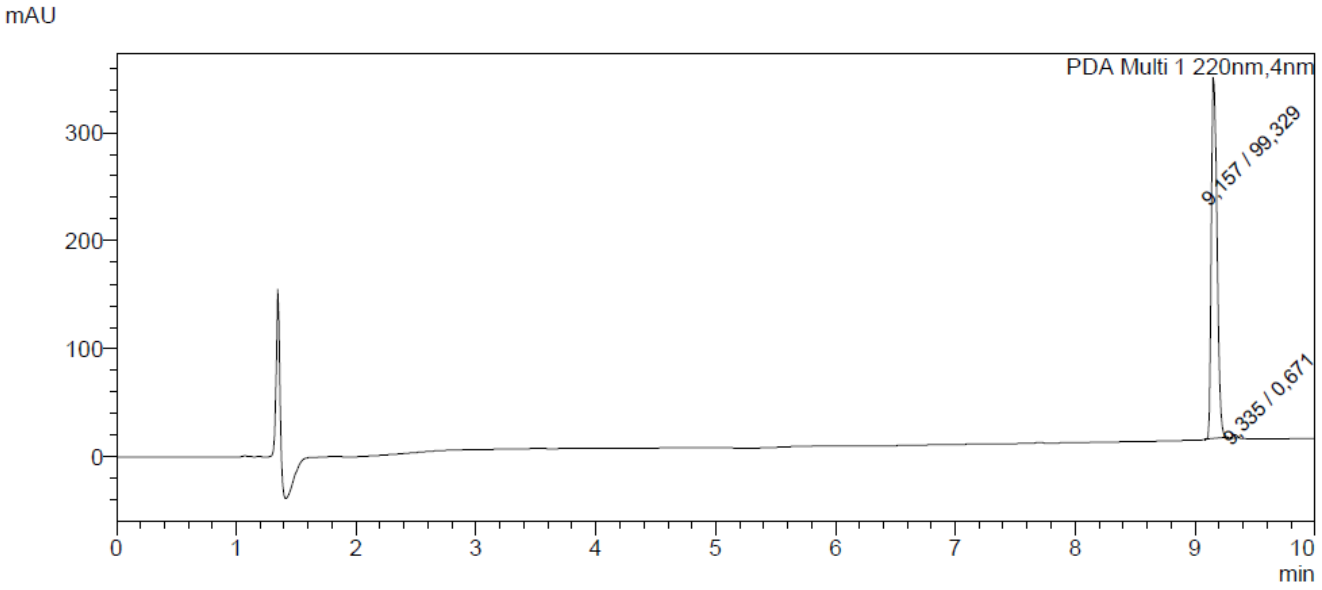
Method Filename : 20_60B_10min_purified2014.lcm
Batch Filename : 2017_11_29_1.lcb
Vial # : 1-79
Injection Volume : 10 uL
Date Acquired : 11/29/2017

Complete Chromatogram



PDA Ch1 220nm

Peak#	Ret. Time	Area%	Height	Area
1	9,157	99,329	334237	1054005
2	9,335	0,671	3021	7117
Total		100,000	337257	1061122

Fig.S22: Compound 6 HPLC

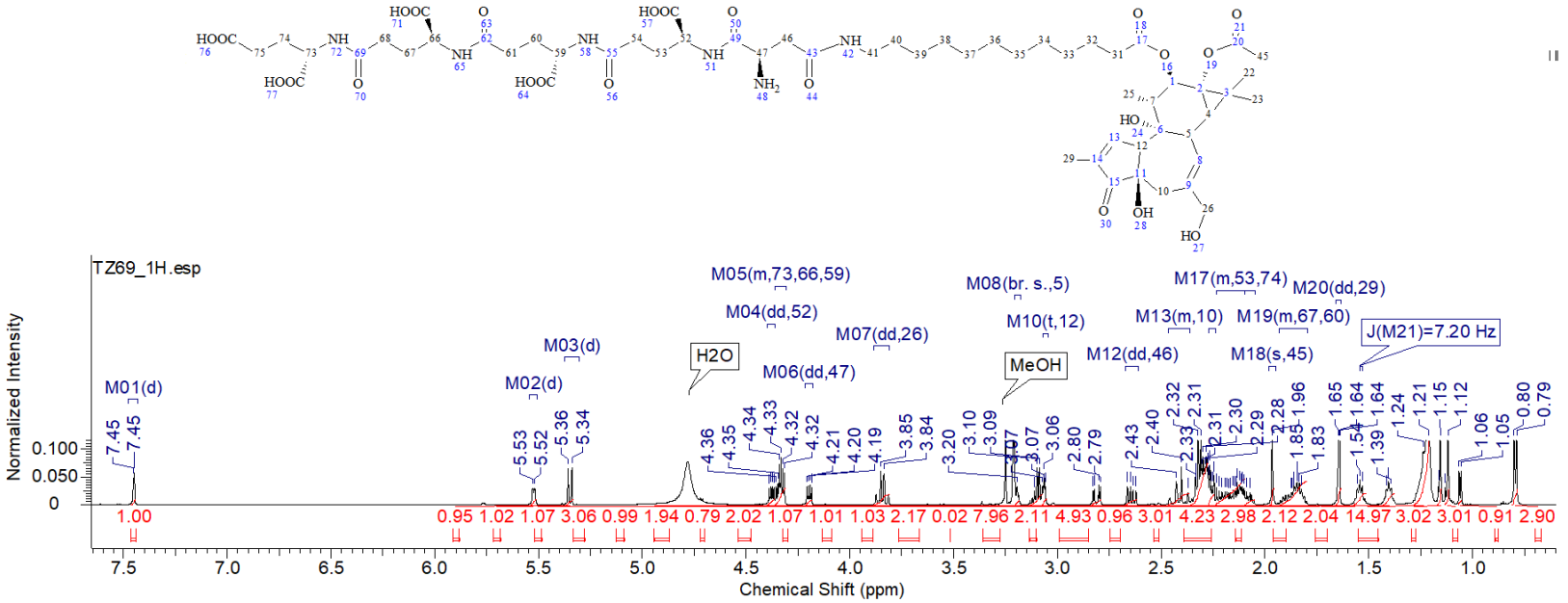


Fig.S23: Compound 6 ^{1}H NMR

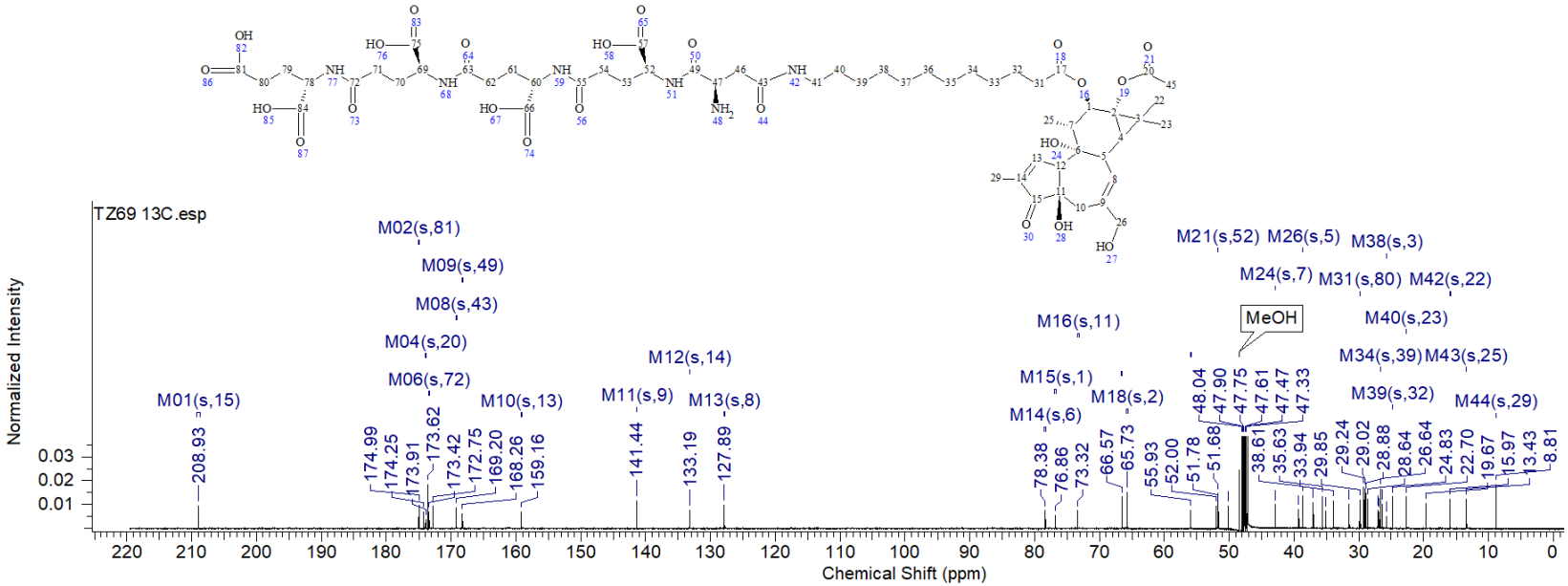


Fig.S24: Compound 6 ^{13}C NMR

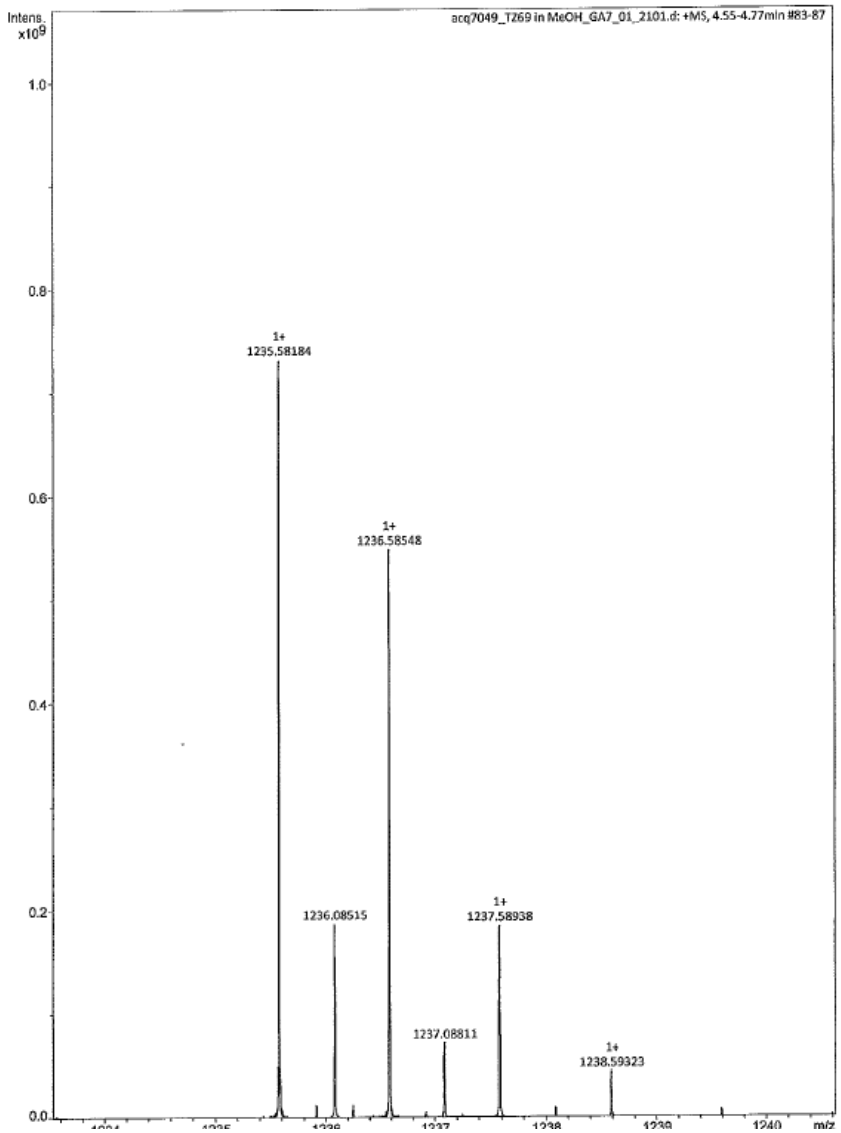


Fig.S25: Compound 6 HRMS

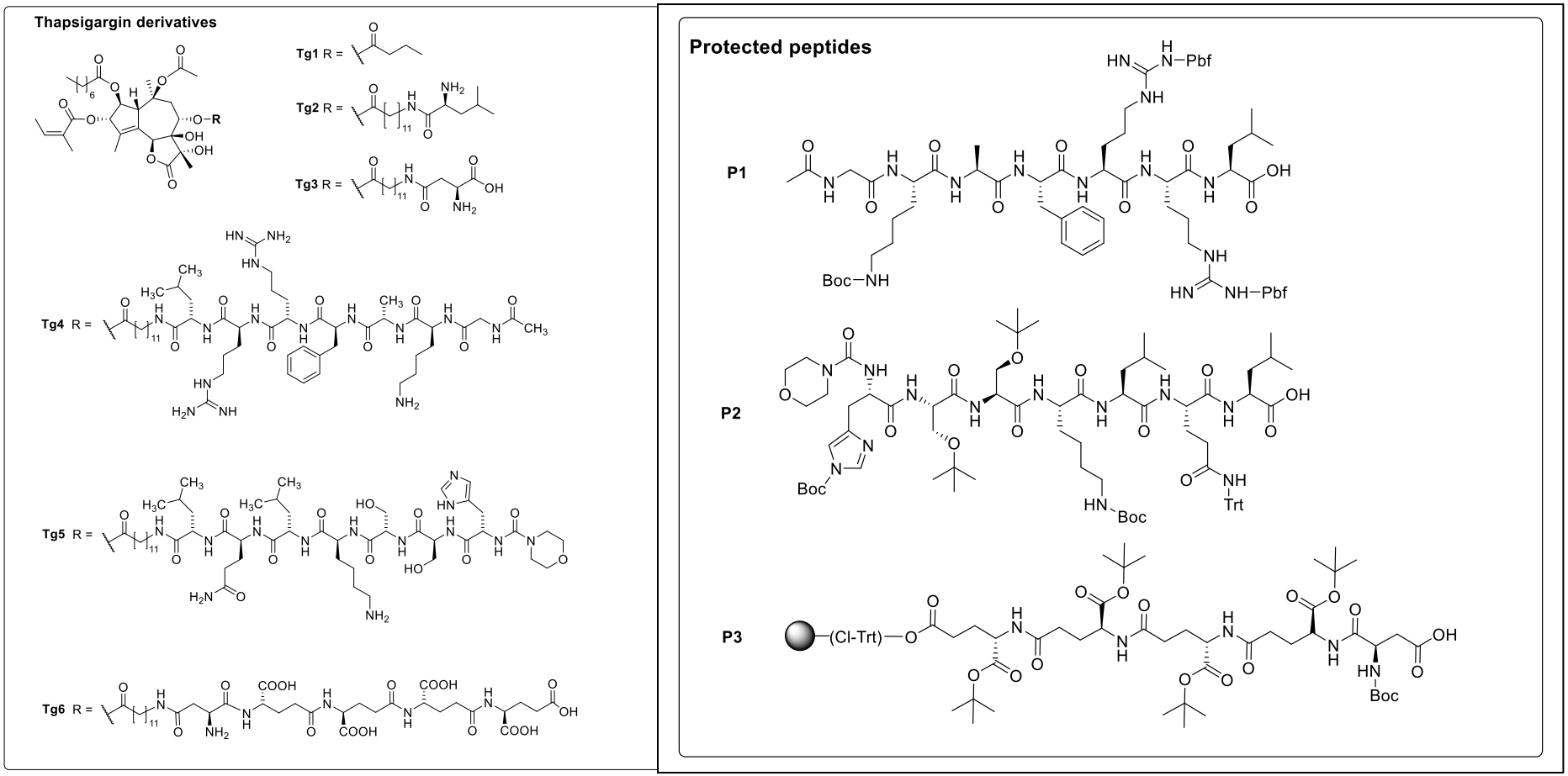


Fig.S26: Prodrugs and drugs of thapsigargin. Tg1 Thapsigargin, Tg2 N-Leu- O-8-12-aminododecanoyl-O-8-debutanoylthapsigargin, Tg3 N-βAsp-O-8-12-aminododecanoyl-O-8-debutanoylthapsigargin, Tg4 G114, prodrug designed for cleavage by hK2, Tg5 G115, prodrug designed for cleavage by PSA, Tg6 G202, mipsagargin, prodrug designed for cleavage by PSMA, P1 protected peptide substrate for hK2, P2 protected peptide substrates for PSA, P3 protected peptide substrate for PSMA.

S.3: Design of Prodrugs of Thapsigargin

In the case of thapsigargin (**Tg1,** Fig. S26) selectivity toward cancer tissue was obtained by conjugation of the toxin with peptides that are substrates for human glandular kallikrein 2, hK2, (prodrug **Tg4**),[8, 9] PSA (prodrug **Tg5**) or PSMA (prodrug **Tg6**, also named mipsagargin).[10] Replacement of the butanoyl group with a 12-aminododecanoyl moiety enabled introduction of an anchoring point for a peptide.[11, 12] Compound **Tg4** contains the peptide Gly-Lys-Ala-Phe-Lys-Arg-Leu, which is a favoured substrate for hK2.[8, 9] The C-terminal Leu is introduced since no cleavage with hK2 was observed in the absence of this amino acid.[13] The hK2 protease is overexpressed in prostate tumours.[8, 12] The peptide sequence His-Ser-Ser-Lys-Leu-Gln-Leu in compound **Tg5** is efficiently cleaved by PSA,[12] while γ-Glu-γ-Glu-γ-Glu-γ-Glu-β-Asp is cleaved by PSMA.[10] Again, the C-terminal amino acids (Leu and β-Asp, respectively) were introduced to make the prodrugs substrates for the enzymes. Enzymatic cleavage of the prodrugs affords the active compounds (**Tg2** or **Tg3**).[10, 12, 14] The peptides in **Tg4** and **Tg5** are capped with acetyl and morpholine groups, respectively, in order to increase solubility. Mipsagargin has successfully passed clinical trial 2[15]

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