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Enantioselective syntheses and application of 4-*epi*-galiellalactone and the corresponding activity-based probe: from strained bicycles to strained tricycles†

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The [6,5,5] tricyclic fungal metabolite galiellalactone is a Michael acceptor that has been demonstrated to be a covalent inhibitor for Signal Transducer and Activator of Transcription 3 (STAT3). Recognizing the ring strain associated with the skeleton of this natural product, we utilized 1*R*-5*S*-bicyclo[3.1.0]hexan-2-one as the starting material and developed two novel approaches to accomplish the enantioselective total synthesis of the C4 epimer of galiellalactone in 5 and 7 steps, respectively, which capitalized on an efficient radical cyclization/fragmentation cascade reaction. Furthermore, an activity-based probe of 4-*epi*-galiellalactone with a terminal alkyne tag was successfully prepared to enable the experiments of activity-based protein profiling (ABPP). Through western blot and proteomic analysis, we not only confirmed the known target STAT3, but also identified a new target protein ataxin-7, which formed a covalent bond with the probe in intact cells *via* the Cys-129 residue.

Introduction

Small molecule natural products with electrophiles reacting with specific nucleophilic protein residues are extremely valuable tools for biomedical research, and have led to important discoveries in life sciences.[1] Based on the structural features, the majority of protein-reactive natural products could be divided into two groups. One contains strained ring systems including epoxides, β-lactams and β-lactones, and the release of ring-strain drives the ring-opening reaction with the protein residue, while the other group are Michael acceptors.[2] Both groups, which are well honed by the coevolution of natural products and their macromolecular targets, have provided numerous inspirations not only for the development of covalent inhibitors but also for activity-based protein profiling (ABPP) technology.[3] It is noteworthy that nature also takes advantage of the ring strain to enhance the reactivity of Michael acceptors. One of the most famous examples is wortmannin, a potent and selective PI3K inhibitor, which possesses a strained energy of ~12.1 kcal mol^{-1}.[4] Interested in the organic synthesis and chemical biology of natural product Michael acceptors with strain-enhanced reactivity, we have recently reported the syntheses of (+)-wortmannin and maoecrystal P, a highly oxidized *ent*-kauranoid.[5] Herein, we disclose our work in the chemotype of galiellalactone (**1**, Fig. 1), a fungal metabolite first isolated from *Galiella rufa* that embeds an α,β-unsaturated ester in the [6,5,5] tricyclic skeleton.[6]

Galiellalactone has been reported to possess a broad spectrum of biological activities, including antitumor,[7] anti-inflammatory[8] and anti-HIV[9] properties. In particular, the natural

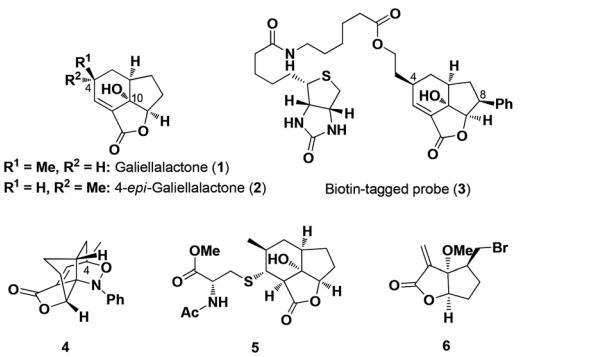

Fig. 1 Structures of natural product (−)-galiellalactone and some of its bioactive analogues.

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†Electronic supplementary information (ESI) available: Tables S1–5, Fig. S1–5, detailed experimental procedures, compound characterization data, and CIF for **2**. CCDC 1860243. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c8ob01915k

product significantly inhibits the growth of androgen-insensitive prostate cancer cells including the stem cell-like ALDH-positive cells both *in vitro* and *in vivo*.[7b] Besides combating castration-resistant prostate cancer, galiellalactone has showed promise in both allergic asthma[8a] and atherosclerosis[8b] mouse models. In terms of the mechanism-of-action, it has been suggested that galiellalactone targets the Signal transducer and activator of transcription 3 (STAT3), a critical transcription factor that is persistently activated in a variety of human cancers.[10] Using a biotinylated probe **3** developed by Sterner's group, Hellsten and co-workers demonstrated that galiellalactone reacted with several cysteine residues of the protein to block STAT3–DNA interaction independent of the STAT3 phosphorylation status.[11] The probe molecule **3** also facilitated the investigation by Muñoz's group, which revealed that galiellalactone could directly target the p65 subunit of NF-κB and impair the nuclear translocation of this transcription factor.[9]

Structure–activity relationship (SAR) studies of galiellalactone have been conducted by several groups regarding its antitumor or STAT3-inhibitory activity.[12–17] In general, the Michael acceptor system together with the heteroatom substitution at C10 is essential for the activity.[12] It has also been reported that the C4-epimer **2** is equally active compared to (−)-galiellalactone (**1**).[13,14] However, extending side chains at C4 significantly reduced its ability to inhibit the proliferation of tumor cells, whereas biological activity was restored when a phenyl group was added at C8 (probe **3** as an example).[15] It is noteworthy that oral administration of a prodrug conjugate **5** prepared by the addition of a cysteine derivative to galiellalactone demonstrated much better pharmacokinetic properties than those of the parent natural product on a mouse model.[16] Most recently, Suh and co-workers found that truncation of the cyclohexene moiety of galiellalactone to [3.3] bicyclic lactone (**6** as an example) improved the cytotoxic effects against triple-negative breast cancers (TNBCs).[17]

Based on a comparison of the relative differences in the energy of hydrogenation of **7** and **9**, we estimated that the strain energy of the galiellalactone skeleton is around 4 kcal mol^{-1} (Fig. 2A, see the ESI† for details), which might play a vital role in the reactivity of this unique natural product. Furthermore, the tendency of a Michael acceptor to undergo nucleophilic addition could be estimated by its LUMO energy. The LUMO energies of **7** and **9** are close to those of methyl acrylate and methyl vinyl ketone, respectively; the LUMO energy gap around 0.28 eV between the bicycle **7** and the strained tricycle **9** may endow the latter with higher activity to undergo nucleophilic addition with residues of targeting proteins (Fig. 2B, see the ESI† for details).

Intrigued by the inherent chemical reactivity of the strained tricyclic Michael acceptor observed in galiellalactone, we aimed at preparing an appropriate activity-based probe and using the powerful mass spectrometry-based proteomic profiling to shed light on the corresponding proteinaceous reaction partners under physiologically relevant conditions in addition to STAT3 and NF-κB. Furthermore, positive results from previous SAR studies[15–17] argue for the urgency of developing a new and concise synthesis to access diversified analogues and expand the research territory of such an important natural product.

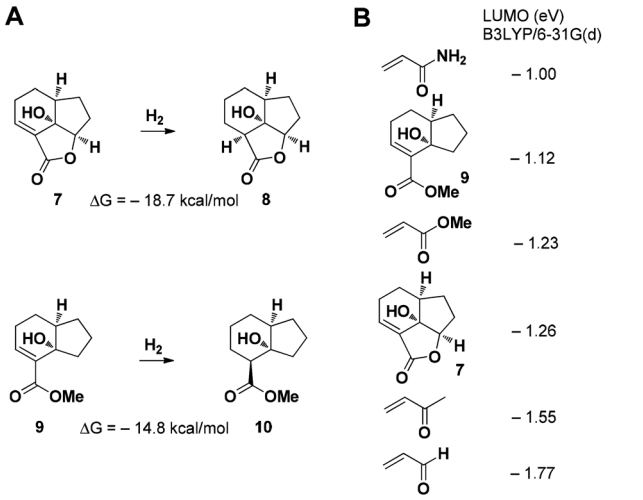


Fig. 2 (A) Approximate reaction Gibbs free energy determined at the B3LYP/6-31G(d) level in Gaussian09. (B) The LUMO energy of various Michael acceptors determined at the B3LYP/6-31G(d) level in Gaussian09.

Three total syntheses of galiellalactone have been reported so far. Sterner and co-workers achieved the first total synthesis of (+)-galiellalactone starting from (+)-pulegone, in which the lactone ring and the tertiary hydroxyl group at C10 were furnished simultaneously by the opening of an epoxide embedded in a bicyclic intermediate.[18] Later on, Sterner's group deployed a palladium-catalyzed tandem reaction followed by Diels–Alder cyclization to efficiently access the same bicyclic intermediate, thus completing the syntheses of the target molecule and its analogs, including the aforementioned probe **3**.[14] In comparison, the latest synthesis by Suh and co-workers relied on an intramolecular palladium-catalyzed allylic alkylation and olefin metathesis to construct the tricyclic ring system.[19] Besides the *de novo* chemical synthesis, the biosynthetic pathway[20] of **1** inspired Sterner's group to combine the synthesis of desoxygaliellalactone with the enzymatic hydroxylation at C10 for the production of (−)-galiellalactone,[21] whereas Lebel's group completed a formal synthesis by developing a one-pot copper-catalyzed methylenation-Diels–Alder reaction.[22]

Results and discussion

Retrosynthetic analysis

Our recent syntheses of (−)-hibiscone C and lysergine based on the radical cyclization/fragmentation strategy inspired a novel disconnection of 4-*epi*-galiellalatone (**2**) (Fig. 3),[23] which has been reported to exhibit a similar activity to that of the aforementioned natural product **1**.[13,14] The late-stage intermediate **11** could not only provide **2** *via* reduction, but also be

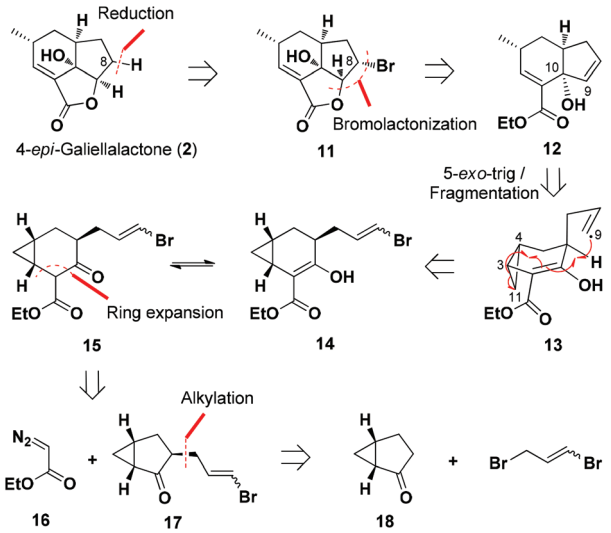
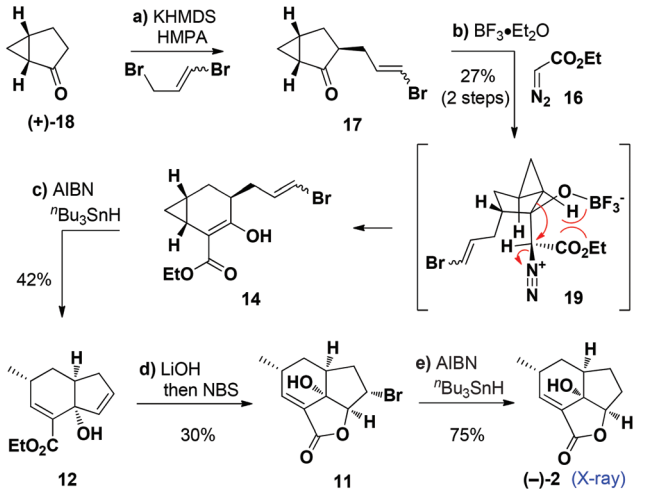


Fig. 3 Retrosynthetic analysis of 4-*epi*-galiellalactone (**2**).

converted to an activity-based probe by introducing a terminal alkyne tag for click chemistry conjugation. While **11** could be obtained from bicycle **12** *via* bromolactonization, we hypothesized that **12** would be prepared in one step from bromide **14** *via* the radical cyclization/fragmentation cascade involving vinyl radical **13** as the intermediate. Due to stereoelectronic effects, ring-opening of the cyclopropane would take place predominately by the scission of the axial C3–C11 bond.[24] The keto-tautomer of **14**, ketoester **15**, would be afforded by a regioselective ring expansion of **17**. Finally, ketone **17** could be obtained by the allylic alkylation of chiral bicycle **18** in a stereoselective manner given the steric hindrance of the fused cyclopropane ring. In comparison with previous syntheses that introduced the tertiary hydroxyl group at C10 through the opening of epoxide,[14,18] allylic oxidation[19] or enzymatic hydroxylation,[21] our strategy traced this motif back to the carbonyl group of substrate **18**.

Synthesis of 4-*epi*-galiellalactone

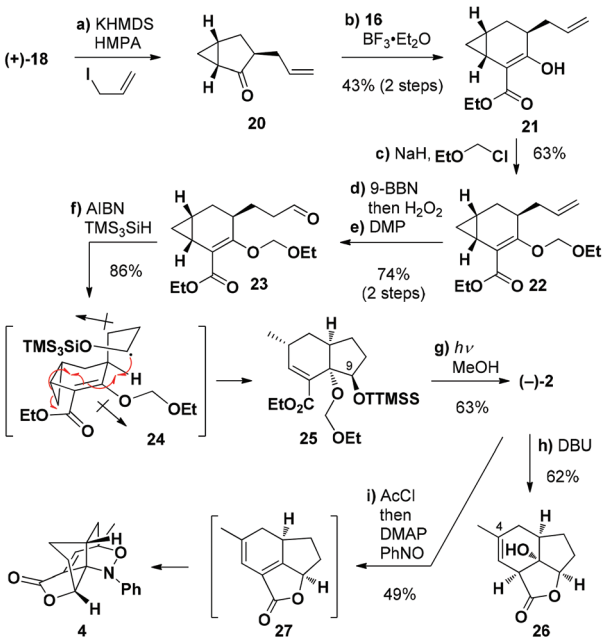
We commenced our studies by preparing chiral (+)-**18** according to known procedures on a multi-gram scale (Scheme 1).[25] The alkylation of **18** with 1,3-dibromo-2-propene afforded ketone **17** as a mixture of *Z*- and *E*-isomers. After substantial optimization, treatment of ketone **17** with ethyl diazoacetate **16** and boron trifluoride etherate at room temperature accomplished the one-carbon ring expansion, leading to compound **14** as the only isolable product. The well-known trend of the preferential migration aptitude in the insertion reaction of a ketone by ethyl diazoacetate is the less substituted or less bulky side.[26] In our scenario, the addition of ethyl diazoacetate **16** to the ketone would take place from the side opposite to the cyclopropane, and therefore the cyclopropyl carbon could be considered as the less bulky side. The transition state **19** with minimized *gauche* steric repulsions was therefore preferred, in which the cyclopropyl carbon was anti-



Scheme 1 First generation synthesis of (–)-4-*epi*-galiellalactone. Reagents and conditions: (a) KHMDS (1.04 equiv.), HMPA, 1,3-dibromo-1-propene (1.5 equiv.), THF, –78 °C; (b) $BF_3{\cdot}Et_2O$ (2 equiv.), **16** (2 equiv.), toluene, 0 °C to rt, 27% (2 steps); (c) $^nBu_3SnH$ (2 equiv.), AIBN (0.25 equiv.), benzene, 80 °C, 42%; (d) $LiOH{\cdot}H_2O$ (1.5 equiv.), THF/H_2O, rt, then $NaHCO_3$ (2 equiv.), NBS (1.2 equiv.), DCM, 0 °C, 30%; and (e) $^nBu_3SnH$ (5 equiv.), AIBN (1.8 equiv.), benzene, 80 °C, 75%. AIBN = azobisisobutyronitrile, DCM = dichloromethane, HMDS = hexamethyldisilazide, HMPA = hexamethylphosphoramide, NBS = *N*-bromosuccinimide, rt = room temperature, THF = tetrahydrofuran.

planar to the leaving group $-N_2^+$, hence giving rise to **14** as the major product. Fortuitously, the enol-tautomer (**14**) was significantly favored over the keto-tautomer (**15**) at room temperature as indicated by NMR spectra (see the ESI†), which could be used directly in the next step and avoid the protection maneuver. To our delight, the desired cyclization/fragmentation reaction took place smoothly in the presence of $^nBu_3SnH$ and AIBN at 80 °C, affording **12** as the final product in 42% yield. To formulate the tricyclic ring, we employed the widely used bromolactonization approach on pentenoic acid-type substrates.[27] A series of conditions were screened using the *in situ* generated carboxylate from **12** as the substrate (Table S3†), but the desired product **11** was isolated in only 30% yield, for which we hypothesized that the strained nature of the [6,5,5]-tricycle played a role. Nonetheless, debromination of **11** under radical conditions provided **2**, the structure of which was determined unambiguously using X-ray crystallography. Without using any protecting groups, our first generation approach delivered (–)-4-*epi*-galiellalactone (**2**) in 5 steps starting from **18** with an overall yield of 2.1%, which prompted us to develop the second generation of synthesis with higher efficiency.

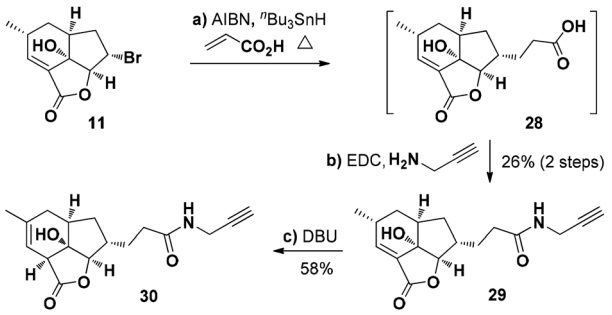
To circumvent the low-yielding bromolactonization step in the initial route, we envisioned a ketyl radical cyclization approach[28] to directly introduce the hydroxyl group at C9 (Scheme 2). Following similar transformations to those of the first route, allylation of bicycle **18** with allyl iodide and subsequent ring expansion using ethyl diazoacetate **16** afforded **21** in 43% yield (2 steps), again as an enol tautomer. In the



Scheme 2 Second generation synthesis of (–)-4-*epi*-galiellalactone. Reagents and conditions: (a) KHMDS (1.05 equiv.), HMPA, allyl iodide (8 equiv.), THF, –78 °C; (b) $BF_3{\cdot}Et_2O$ (1.5 equiv.), **16** (1.5 equiv.), toluene, 0 °C to rt, 43% (2 steps); (c) NaH (1.5 equiv.), $EtOCH_2OCl$ (1.5 equiv.), THF, 0 °C to rt, 63%; (d) 9-BBN (3 equiv.), THF, 0 °C; then EtOH, 6 M NaOH, H_2O_2, 0 °C; (e) DMP (1.5 equiv.), $NaHCO_3$ (4 equiv.), DCM, 0 °C to rt, 74% (2 steps); (f) TMS_3SiH (2 equiv.), AIBN (0.25 equiv.), toluene, 85 °C, 86%; (g) *hv*, MeOH, 63%; (h) DBU (5 equiv.), toluene, 80 °C, 62%; and (i) AcCl (35 equiv.), CH_3CN, rt; then PhNO (10 equiv.), DMAP (1 equiv.), CH_3CN, rt, 49%. 9-BBN = 9-borabicyclo[3.3.1]nonane, DMP = Dess–Martin periodinane, DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, TMS = trimethylsilyl, TTMSS = tris(trimethylsilyl)silyl.

meantime, a cursory screen of Lewis acid and solvent was conducted to increase the efficiency of the latter step (Table S4†). Protection of the hydroxyl group with the ethoxymethyl group followed by hydroboration/oxidation provided aldehyde **23** as the ketyl radical precursor. The desired cyclization/fragmentation reaction took place in the presence of tris(trimethylsilyl)silane and AIBN at 85 °C, affording **25** in 86% yield as a single diastereomer. The preference of forming **25** could be rationalized by invoking the transition state **24** that minimized the dipole–dipole interaction. We were delighted to find that irradiation of **25** in methanol effected the deprotection of both hydroxyl groups followed by lactonization to furnish (–)-**2** in 63% yield in one step. Consequently, the second generation route provided **2** in 7 steps with an overall yield of 11%.

Attempts to epimerize the C4 methyl groups under various conditions were not successful. But we accidentally obtained **26** with shifted double bonds by treating **2** with DBU,[29] which could be used as a negative control in our follow-up studies due to the lack of active Michael acceptors. In a one-pot procedure, **2** was further converted to the tetracyclic compound **4**, a molecule previously prepared from **1** and reported to have comparable STAT3 inhibitory activity.[12]



Scheme 3 Syntheses of the small-molecule probe **29** and the pseudo-probe **30**. Reagents and conditions: (a) acrylic acid (10 equiv.), $^nBu_3SnH$ (2 equiv.), AIBN (0.5 equiv.), toluene, 80 °C; (b) 2-propynylamine (2 equiv.), EDC·HCl (2 equiv.), DMAP (0.2 equiv.), Et_3N (4 equiv.), DCM, rt, 26% (2 steps); and (c) DBU (5 equiv.), toluene, 80 °C, 58%. EDC = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide.

Synthesis of the activity-based probe (Scheme 3)

The reaction of bromide **11** with excess acrylic acid in the presence of $^nBu_3SnH$ and AIBN at 80 °C led to a crude product of acid **28**, which was directly subjected to amidation reaction with propargylamine to afford **29** as our probe molecule. Though the yield was low (26% over 2 steps), the expedient approach still provided us enough quantities of **29**. Furthermore, we treated **29** with DBU to effect the olefin isomerization to prepare a pseudo-probe (**30**), which could be used as a negative control of the Michael acceptor probe **29** in ABPP studies.

Activities of synthesized galiellalactone analogues against cell proliferation

The anti-proliferation effects of galiellalactone and its analogues have been extensively studied in prostate cancer cells and recently in breast cancer cells,[7,12–17] whereas we found that **1** also demonstrated significant cytotoxicity in two human T-cell acute lymphocytic leukemia (T-ALL) cell lines (MOLT-4 and Jurkat), which were then used for our subsequent investigation (Table 1; see Fig. S1† for dose–response curves). The potency of 4-*epi*-galiellalactone (**2**) was similar to that of

Table 1 The anti-proliferation effects of (–)-galiellalactone and synthesized analogues against T-ALL cell lines MOLT-4 and Jurkat[a]

	IC_{50} (μM)	
Compounds	MOLT-4	Jurkat
GL (**1**)	1.5	1.5
4-*epi*-GL (**2**)	2.0	5.8
Probe (**29**)	8.6	23
26	>100	>100
Pseudo-probe (**30**)	>100	>100
12	>100	>100
11	14	4.0
4	6.9	5.8

[a] The inhibitory effects of compounds on the proliferation of T-ALL cells were determined using the MTT assay after 72 h of treatment.

galiellalactone (**1**) (IC_{50}s between 2 and 4 fold), while probe **29** showed four-fold decreased cytotoxicity in MOLT-4 cells (IC_{50} = 8.6 μM). In contrast, **26** and **30** with shifted double bonds hardly had any anti-proliferation activity (IC_{50} > 100 μM), suggesting the critical role of the Michael acceptor system. Interestingly, the bicyclic compound **12** did not show any activity (IC_{50} > 100 μM) in spite of the intact α,β-unsaturated ester in the molecule. We hypothesized that the absence of the strain energy conferred by the [6,5,5] tricyclic skeleton decreased the reactivity of the Michael acceptor in **12**, which significantly diminished its bioactivity. Moreover, bromide **11** and the Diels–Alder adduct **4** also exhibited moderate anti-proliferation activities.

Moreover, treatment of MOLT-4 cells with 4-*epi*-galiellalactone (**2**) did not affect the expression of p-STAT3(Tyr705) and total STAT3 except those at 25 μM compound concentration (Fig. 4), which was consistent with observations of galiellalactone (**1**) in DU145 cells.[11] The appearance of the apoptotic marker cleaved poly(ADP-ribose)polymerase (PARP) accompanying the increasing concentrations of **2** was significant, while cell cycle regulatory proteins c-Myc and CDK6 were downregulated; in comparison, the expression levels of cyclin D1 and Mcl-1 were not altered.

MS-Based ABPP profiling of 29-interaction proteins

Before carrying out the activity-based protein profiling (ABPP) of probe **29**, we first validated the covalent binding of this probe to STAT3 (Fig. 5A). MOLT-4 or Jurkat cells were treated with probe **29** for 2 h and then lysed. The cell lysates were subjected to the $CuSO_4$-catalyzed click reaction with biotin-azide and then the binding proteins were successively pulled down by streptavidin beads, separated using SDS-PAGE and immunoblotted with STAT3 antibody. Pretreatment of cells with galiellalactone (**1**) or 4-*epi*-galiellalactone (**2**) prior to the addition of **29** significantly reduced the binding of STAT3 to the probe, thus demonstrating a competitive binding mode.

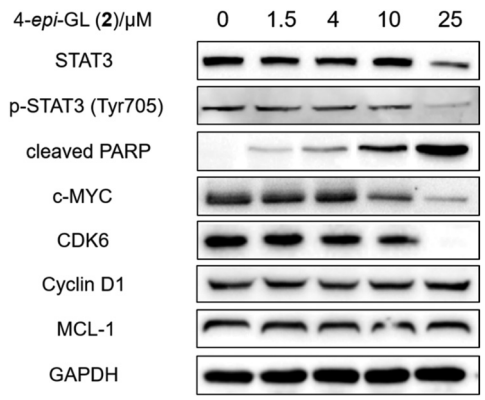


Fig. 4 Western blot analysis of MOLT-4 cells treated with 4-*epi*-galiellalactone (**2**). MOLT-4 cells were treated with **2** for 48 h and then lysed. The cell lysates were resolved using polyacrylamide gel electrophoresis under denaturing conditions, followed by western blot analysis of the indicated proteins.

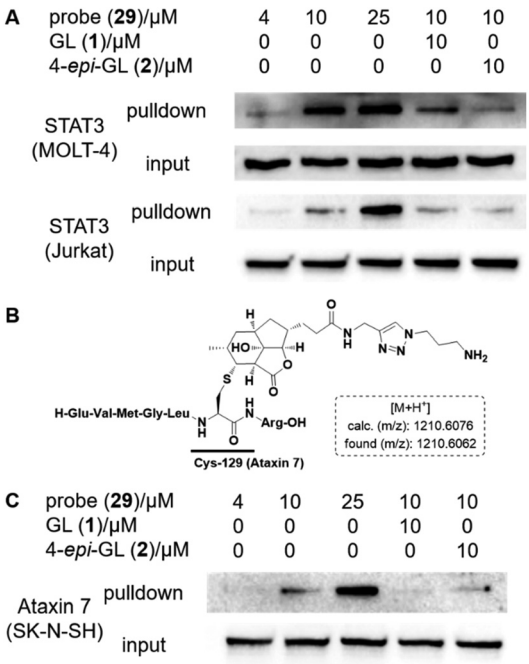


Fig. 5 (A) Western blot analysis of biotin-enriched MOLT-4 or Jurkat cells treated with probe **29**. (B) Trypsin-digest LC-MS/MS analysis indicating modification of ataxin-7 with **29** at residue Cys129. (C) Western blot analysis of biotin-enriched SK-N-SH neuroblastoma cells treated with probe **29**.

In situ proteome labeling was carried out using 25 μM **29** in the absence and presence of **2**, and a photocleavable biotin-azide (Fig. S2†) was used to form a conjugate with the targeting proteins *via* a click reaction, resulting in the removal of the connected biotin by UV light at 365 nm following the pull-down by streptavidin beads.[30] After trypsin digestion, the resulting samples were subjected to LC-MS/MS analysis (see the ESI† for details). Again, STAT3 was clearly identified as a target of **29** (see Table S5† for the list of hit proteins), even though the covalent modification site was not identified. In contrast, the proteome labeling of the pseudo-probe **30** with shifted double bonds turned out to be minimal (see Fig. S3† for silver staining), indicating the necessity of the Michael acceptor motif in the 4-*epi*-galiellalactone probe **29** regarding the reaction with proteins in intact cancer cells.

Importantly, the chemical proteomics approach also allowed for the discovery of target proteins that may not be relevant for the anti-cancer or anti-inflammatory activities intensively studied. From the MS/MS data, we found that ataxin-7, a protein mainly expressed throughout the central nervous system,[31] was selectively labeled by probe **29**. Mutated ataxin-7, with polyglutamine-tract expansion, is associated with a rare neurodegenerative disease named spinocerebellar ataxia type 7 (SCA7) that has no current therapies in the clinic.[32] The proteolysis of this mutated protein produces toxic fragments that underlies the pathogenesis of the disease.[33] To our knowledge, neither small molecules interacting with ataxin-7 nor the targetable cysteines within this protein have

been reported before. The LC-MS/MS data suggested that probe **29** could form a covalent bond with Cys-129 of ataxin-7 (Fig. 5B; see Fig. S4† for MS^2 data). Subsequently, the binding interaction between ataxin-7 and the probe was further demonstrated by western-blot following pull-down experiments using both SK-N-SH neuroblastoma cells (Fig. 5C) and fresh isolated mouse neuro cells (Fig. S5†). Herein, for the first time, we discovered that small-molecule probe **29** could covalently bind ataxin-7, and the labeling event was competed away by both galiellalactone (**1**) and 4-*epi*-galiellalactone (**2**). Whether the biological functions of ataxin-7 are affected invites further investigations.

Conclusions

In summary, we have accomplished the enantioselective total synthesis of 4-*epi*-galiellalactone *via* two approaches in 5 (2.1% overall yield) and 7 steps (11% overall yield), respectively. The key for both syntheses was the application of a radical cyclization/fragmentation strategy, and the three membered ring of the starting material (+)-**18** not only provided excellent stereochemical control but also enabled strain-release fragmentation. Moreover, regioselectivity for the ring enlargement of the strained bicyclic ketones **17** and **20** was also notable. In leukemia cell lines MOLT-4 and Jurkat, 4-*epi*-galiellalactone **2** exhibited cytotoxicity to a similar extent in comparison with the natural product galiellalactone **1**, while the bicyclic intermediate **12** was not active, indicating the critical role of the strained tricyclic skeleton for promoting the reactivity of the Michael acceptor.

Subsequently, the development of the activity-based probe **29** enabled the ABPP experiments, with the pseudo-probe **30** serving as a negative control. Through western blot and proteomic analysis, we confirmed the known target STAT3 and shed light on other potential interacting proteins. One prominent example is the identification of the covalent attachment of **29** to Cys129 of ataxin-7, which has not been targeted by small molecules before. The proteolysis of mutated ataxin-7 produces toxic fragments that underlies the pathogenesis of the neurodegenerative disease SCA7.[33] Therefore, additional studies are urgently needed to elucidate whether the reaction of galiellalactone and its analogues intervenes in the proteolysis of mutated ataxin-7.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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