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Toward Aplyronine Payloads for Antibody-Drug Conjugates: Total Synthesis of Aplyronines A and D

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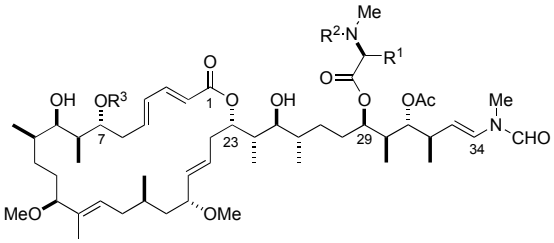
The aplyronines are a family of antimitotic marine macrolides that disrupt cytoskeletal dynamics by dual targeting of both actin and tubulin. Given their picomolar cytotoxicity profile and unprecedented mode of action, the aplyronines represent an excellent candidate as a novel payload for the development of next-generation antibody-drug conjugates (ADCs) for cancer chemotherapy. Enabled by an improved second-generation synthesis of the macrolactone core **5**, we have achieved the first total synthesis of the most potent congener aplyronine D together with a highly stereocontrolled synthesis of aplyronine A. To facilitate step economy, an adventurous site-selective esterification of the C7 hydroxyl group was performed to install the *N,N,O*-trimethylserine pharmacophore to directly afford aplyronines A and D. Toward the assembly of ADCs incorporating an aplyronine warhead, the C29-ester derivative **4** featuring an Fmoc-amino substituted linker attached to the actin-binding tail region was also prepared by adapting this flexible endgame.

Introduction

Natural products offer a rich source of potent bioactive compounds for the development of new drugs, particularly cytotoxic anticancer agents.[1] Their therapeutic window can be enhanced by targeted drug delivery to tumour cells, where natural product derivatives such as the tubulin-binding agents maytansine and monomethyl auristatin E are clinically validated cytotoxic payloads for antibody-drug conjugates (ADCs).[2] Other highly potent anticancer natural products with unique mechanisms of action have potential to be developed as novel ADC payloads, provided they can be obtained in useful quantities and modified to include a suitable linker for bioconjugation.

A promising novel chemotype for targeted delivery by ADCs are the aplyronines (Figure 1). These complex polyketide metabolites were isolated by Yamada and co-workers[3] from the sea hare *Aplysia kurodai*, a gastropod mollusc collected off the coast of the Mie prefecture in Japan. From a molecular architecture perspective, the aplyronines are characterised by a highly substituted 24-membered macrolactone core, with an elaborate side chain terminating in an *N*-methyl-*N*-vinyl formamide moiety and an amino acid residue attached to C29. The most biologically active congeners, as represented by aplyronines A (**1**) and D (**2**), also feature a signature *N,N,O*-trimethylserine residue attached to C7, which is generally present as a mixture of epimers. The configurations of the five alkenes and 17 stereocentres in aplyronine A were determined by detailed spectroscopic analysis in combination with chemical degradation and correlation with fragments prepared by enantioselective synthesis.[4] The resulting stereochemical assignment was validated by the first total synthesis[5] and the X-ray crystallographic analysis of its 1:1 complex with actin.[6] Notably, the eight known members of the aplyronine family differ in the identity of the amino acid at C29 (*N,N*-



1: *aplyronine A*, $R^1 = R^2 = Me$, $R^3 = N,N,O$-trimethylserine
2: *aplyronine D*, $R^1 = H$, $R^2 = Me$, $R^3 = N,N,O$-trimethylserine
3: *aplyronine C*, $R^1 = R^2 = Me$, $R^3 = H$
4: $R^1 = H$, $R^2 = (CH_2)_6NHFmoc$, $R^3 = N,N,O$-trimethylserine

Figure 1 Structures of the aplyronine congeners **1–3** and the linker-modified derivative **4**.

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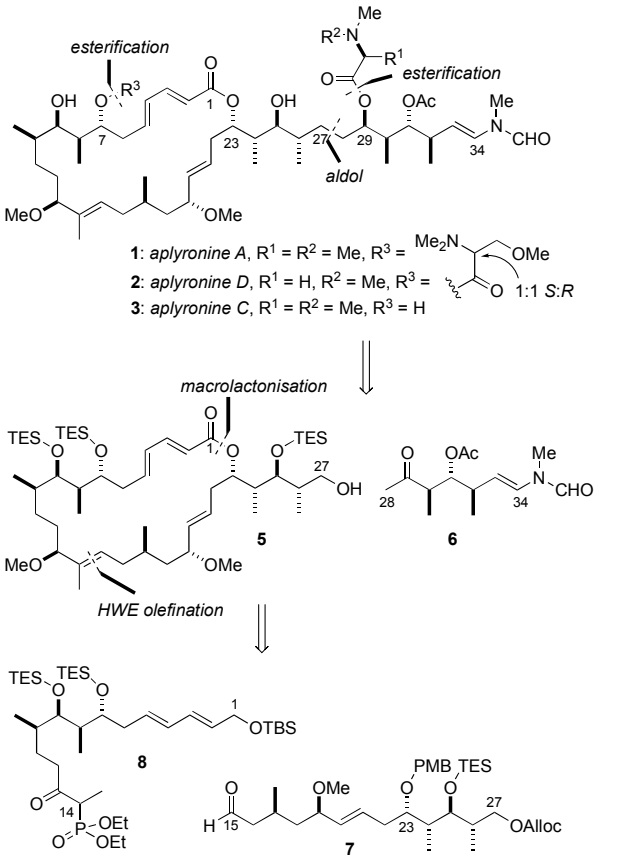
dimethylalanine, N-methylalanine or N,N-dimethylglycine) and whether the N,N,O-trimethylserine is attached at C7 or C9, or omitted altogether. These permutations in the position and identity of the amino acids can lead to marked SAR differences, e.g. aplyronine C (3) lacking the N,N,O-trimethylserine moiety is around two orders of magnitude less cytotoxic than aplyronines A and D.

The aplyronines represent potential clinical candidates in cancer chemotherapy, although there are severe supply issues impacting on their further development. On testing in the US National Cancer Institute primary screen, aplyronine A (NSC687160) displayed impressive sub-nanomolar antiproliferative activity (mean GI_{50} = 0.2 nM) in the 60 cell line panel.[2a] Moreover, the *in vivo* efficacy of aplyronine A was demonstrated against a range of five implanted tumour types in mouse xenograft models, with high survival rates observed for Lewis lung carcinoma and P388 leukaemia.[3a,b] Intriguingly, the minor congener aplyronine D, displaying a structural variation in the amino acid residue at C29, was later reported to possess even greater cytotoxicity than aplyronine A (*e.g.* GI_{50} of 0.071 nM for **2** *vs* 0.45 nM for **1** in the HeLa-S3 cervical cancer cell line).[3c] The aplyronines are known to primarily function as small molecule mimics of actin-binding proteins,[6-8] disrupting actin dynamics by severing F-actin and sequestering G-actin. While the details of the actin-binding interaction of the aplyronines have been well characterised, the exceptional cytotoxicity has recently been shown to arise from interference with protein-protein interactions, through the formation of a heterotrimeric complex of aplyronine A with actin and tubulin, synergistically disrupting cytoskeletal dynamics.[9] Although the precise nature of these interactions and the associated binding site on tubulin of the initially formed actin-aplyronine complex are yet to be determined, this dual protein-targeting mode is unprecedented amongst antimitotic natural products.[10] The combination of exceptional potency and a unique mechanism of action make the aplyronines important lead compounds, both as molecular probes to further explore their interaction with actin and tubulin, and in the context of providing a new class of cytotoxic payload for next-generation ADC development.

Given their extremely scarce natural abundance (e.g. only 2.6 mg of aplyronine D was isolated from a 300 kg collection of *Aplysia kurodai*[3c]) and structural complexity, the aplyronine macrolides constitute compelling and challenging synthetic targets.[11-13] Notably, the total synthesis of aplyronines A, B and C was first achieved by the Yamada group,[5] with an improved second-generation route to aplyronine A subsequently reported by Kigoshi and co-workers.[11] Recently, we accomplished a convergent total synthesis of aplyronine C based on asymmetric aldol chemistry developed in our group, leading to a significantly shorter route.[12a] Moving forward, we aimed to devise a flexible and practical synthetic strategy to access the most potent aplyronine congeners and designed analogues for future application as ADC payloads. We now report an expedient total synthesis of both aplyronines A and D *via* a late-stage divergence, together with a linker-modified derivative **4** for planned antibody conjugation, and the development of an improved route to the pivotal macrolactone core.

Results and discussion



Scheme 1 Retrosynthetic analysis highlighting the key fragments **5**–**8**.

As outlined in Scheme 1, our proposed unified synthesis plan for the aplyronines was focused on the controlled construction and assembly of the key fragments **5**–**8**, resulting from disconnections at C27–C28 and C14–C15, and opening of the macrolactone ring. Importantly, it was considered essential to be able to site-selectively install the pharmacophoric *N,N,O*-trimethylserine residue at C7. To simplify the protecting group strategy for enhanced step economy, an adventurous esterification onto the C7 alcohol in aplyronine C (**3**) was proposed to directly afford aplyronine A (**1**). To further streamline the route, we elected to set out by having all three secondary hydroxyl groups at C7, C9 and C25 in the macrolactone core **5** protected as TES ethers. Building on our first-generation route,[12a] the full aplyronine side chain would then be installed by a late-stage aldol coupling[14] to forge the C27–C28 bond between the derived C27 aldehyde and the methyl ketone **6**.

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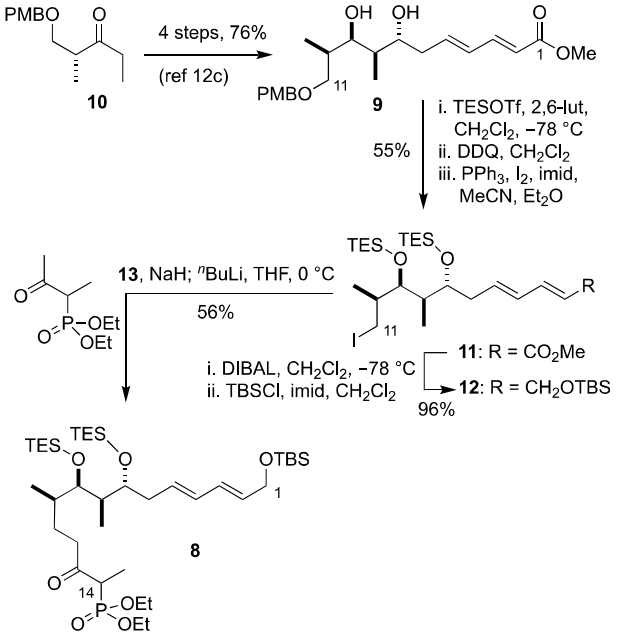
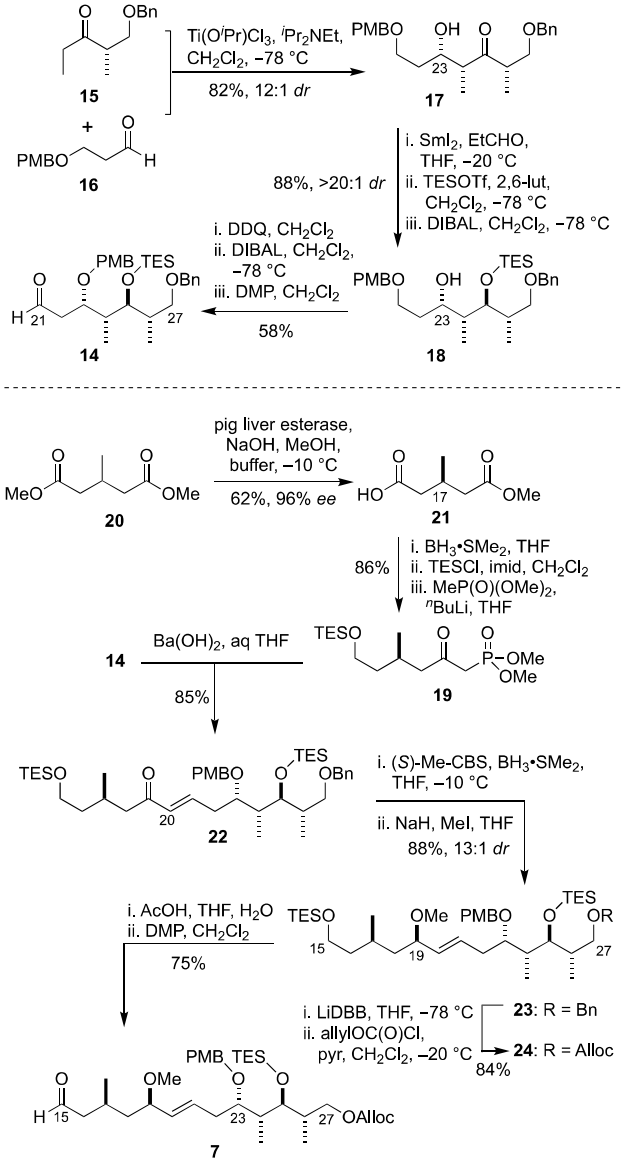
In our previous work, the macrolactonisation of an intermediate 23,25-dihydroxy acid had generated exclusively the incorrect ring size,[12b] which was followed by Ti(O^{i}Pr)$_4$-mediated isomerisation to give a 3:1 mixture in favour of the desired 24-membered macrolactone. The resulting requirement for repeated chromatographic separation and re-equilibration of the isomeric 26-membered macrolactone impacted on material throughput. To circumvent this difficulty, we now planned to achieve a site-specific macrocyclisation step to give solely the desired 24-membered macrolactone. Hence, the C23 hydroxyl would be orthogonally protected as its PMB ether in the C15–C27 aldehyde **7**. To connect the southern and northern fragments, we envisaged that this aldehyde would engage in an (*E*)-selective HWE-type coupling with a revised C1–C14 phosphonate **8**.

Following this flexible and modular plan, we commenced our synthesis campaign with the preparation of the northern fragment **8** (Scheme 2). In practice, this was obtained from the 1,3-diol **9**, incorporating the correctly configured C7–C10 stereotetrad, which was readily prepared on a multigram scale (see the ESI) by our previously described boron-mediated aldol route[12c] from the chiral ketone **10**. The diol **9** was first converted into the *bis*-TES ether (TESOTf), followed by cleavage of the PMB ether (DDQ) and transformation of the resulting alcohol (Ph$_3$P, I$_2$, imid.) into the iodide **11** (55%, 3 steps). Adjustment of the oxidation state at C1 (DIBAL) and silylation of the resulting alcohol (TBSCl) then afforded **12**, which was alkylated at C11 with the dianion (NaH, ^{n}BuLi) of **13**[15] to deliver the desired β-keto phosphonate **8** (54%, 3 steps) accompanied by a minor regioisomer that was best removed after fragment coupling.



Scheme 2 Synthesis of the C1–C14 northern fragment **8**.

The required southern fragment **7** for the planned HWE-type fragment coupling with the phosphonate **8** was assembled from the C21–C27 aldehyde **14** (Scheme 3). Installation of the stereotetrad region of **14** commenced with a titanium-mediated aldol reaction between the chiral ethyl ketone **15**[16] and aldehyde **16**. High diastereoselectivity for the desired *syn*-adduct **17** (82%, 12:1 *dr*) was achieved by employing Ti(O^{i}Pr)Cl$_3$ as the Lewis acid[17] to ensure efficient chelation by the benzyl ether of **15** in the proposed bicyclic aldol transition state. This procedure proved robust on a multi-gram scale, affording greater diastereoselectivity and operational simplicity than the previously employed Sn(OTf)$_2$-promoted aldol reaction.[12c,18] Next, an Evans-Tishchenko reduction[19] (SmI$_2$, EtCHO) of the β-hydroxy ketone **17** efficiently configured the C25 stereocentre (93%, >20:1 *dr*), and the resulting alcohol was



Scheme 3 Synthesis of the C15–C27 southern fragment **7**.

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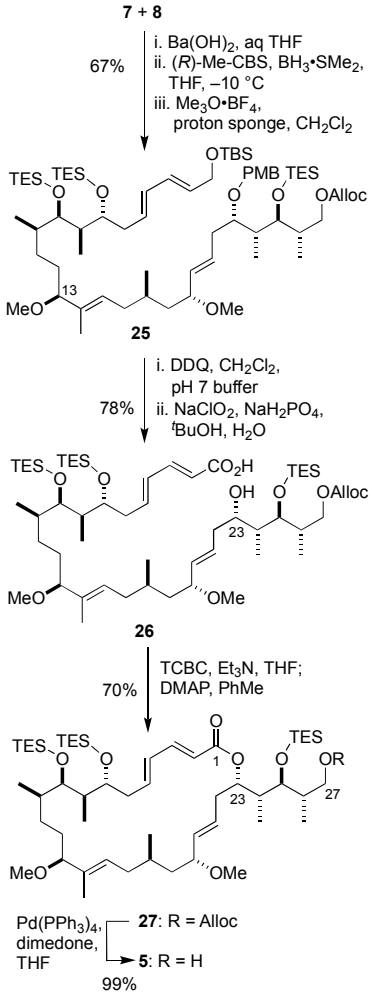
converted into its TES ether (TESOTf), followed by ester cleavage (DIBAL) to afford the secondary alcohol **18** (95%, 2 steps). As dictated by our site-specific macrolactonisation plan, the PMB ether at C21 in **18** was at this point transferred over to the C23 position *via* reductive opening (DIBAL) of the derived PMP acetal (DDQ), generating a primary alcohol for Dess–Martin oxidation[20] to give the C21–C27 aldehyde **14** (58%, 3 steps). Moving forward, elaboration to the C15–C27 fragment **7** was dependent on an HWE olefination involving the β-keto phosphonate **19** containing the isolated C17 methyl-bearing stereocentre. This chiral building block was conveniently accessed through a scalable four-step sequence, by first employing a pig liver esterase-catalysed hydrolysis of diester **20** to give the acid **21** (96% *ee*).[21] Following selective reduction of the acid to the primary alcohol ($BH_3{\cdot}SMe_2$) and TES ether formation, the ester was converted ($MeP(O)(OMe)_2$, ^{n}BuLi) into the β-keto phosphonate **19**. A $Ba(OH)_2$-mediated HWE coupling[22] between **19** and **14** then proceeded smoothly to yield the (*E*)-enone **22** (85%). Asymmetric reduction using the (*S*)-Me-CBS catalyst and $BH_3{\cdot}SMe_2$[23] and methylation (MeI, NaH) of the resulting alcohol next installed the C19 methoxy-substituted stereocentre in **23** (88%, 13:1 *dr*).



Scheme 4 Synthesis of the macrolactone core **5**.

At this stage, the required cleavage of the C27 benzyl ether in the presence of a PMB ether, primary TES ether and alkene in **23** proved challenging, but could be accomplished by controlled treatment with lithium 4,4'-di-*tert*-butylphenylide (LiDBB) at low temperature.[24] The primary alcohol was then converted into its Alloc derivative (allyl chloroformate, pyr) to give **24** (84%, 2 steps). Selective cleavage of the primary TES ether (AcOH) and Dess–Martin oxidation then afforded the desired C15–C27 southern fragment **7** (75%, 2 steps). This scalable sequence could be comfortably carried out to prepare multi-gram quantities of the aldehyde **7** in a single batch.

The stage was now set to explore our planned fragment coupling and macrocyclisation strategy (Scheme 4). Firstly, a similar sequence of $Ba(OH)_2$-mediated HWE olefination, (*R*)-Me-CBS asymmetric reduction and methylation ($Me_3O{\cdot}BF_4$) served to unite the key fragments **7** and **8**, setting in place the required (*E*)-trisubstituted alkene and the C13 methoxy-bearing stereocentre in **25** (67%, 3 steps, 15:1 *dr*). Next, treatment of **25** with DDQ served to excise both the C1 TBS and C23 PMB ethers with concomitant oxidation to the dienal.[25] Further oxidation to the *seco*-acid **26** (78%, 2 steps) was then performed using sodium chlorite. Gratifyingly, the critical macrocyclisation of **26** proceeded smoothly under Yamaguchi conditions[26] (TCBC, Et_3N, DMAP) to deliver the desired 24-membered macrolactone core **27** (70%). Finally, treatment of **27** with $Pd(PPh_3)_4$ and dimedone cleanly removed the C27 Alloc group to intercept the known macrocyclic intermediate **5** (99%),[12a] thus demonstrating the viability of this improved second-generation route.

Moving forward from this key intermediate, we first targeted the synthesis of aplyronines A (**1**) and D (**2**) which differ in the amino acid residue attached at C29 *via* a late-stage divergence (Scheme 5). Following our earlier work,[12a,14] the full side chain was first installed using a boron-mediated aldol reaction (*c*-Hex_2BCl, Et_3N) between the C27 aldehyde **28**, obtained by Swern oxidation of **5**, and the methyl ketone **6**. The resulting adduct was treated with Burgess reagent[27] to effect the controlled β-elimination of the newly formed C27 alcohol to give the (*E*)-enone **29** (65%). This aldol/dehydration sequence proved remarkably effective, particularly considering the presence of a potentially labile acetoxy group at the β' position. Conjugate reduction of **29** could then be achieved with a catalytic amount of copper(I) hydride, following an adaptation of a Lipshutz protocol.[28,29] Diastereoselective reduction ($Zn(BH_4)_2$) of the resulting ketone then afforded the correctly configured C29 alcohol **30** (56%, 2 steps, 10:1 *dr*). We rationalise this useful result based on the participation of an intermediate eight-membered zinc chelate involving the ketone and acetyl carbonyl groups.[30] Strategically, this advanced intermediate **30** represented the divergence point from which the majority of the known aplyronine congeners[3c] and designed analogues might be accessed.

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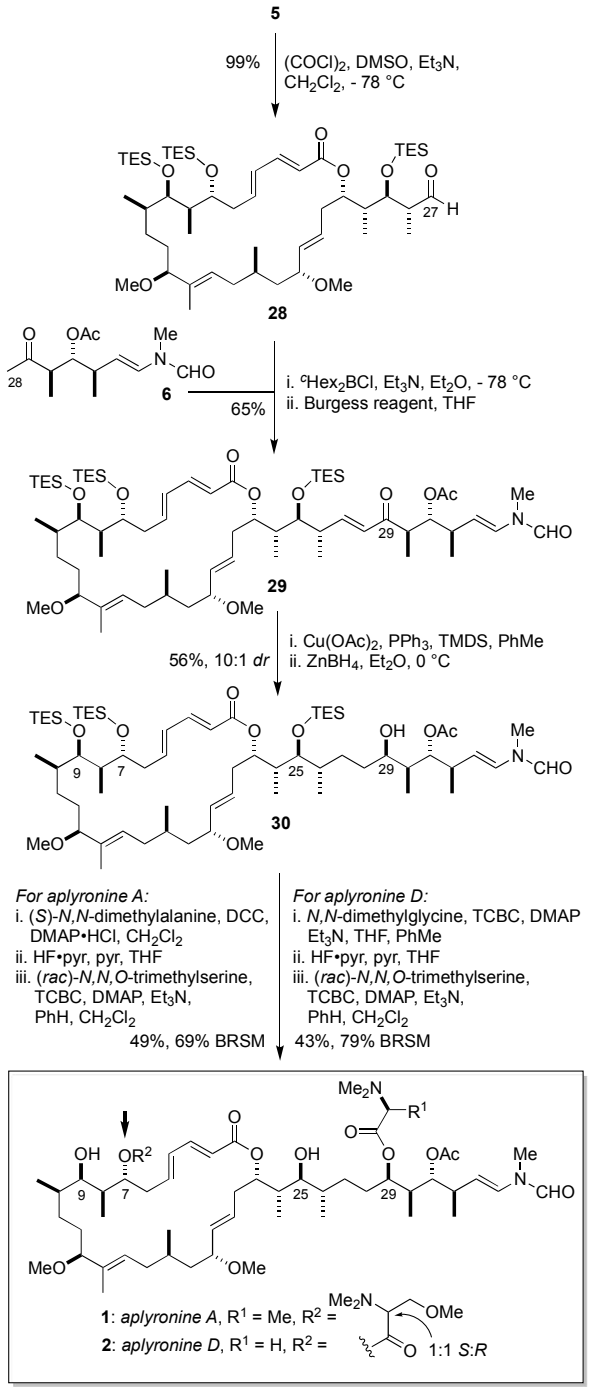
Initially targeting the synthesis of aplyronine A (**1**) from **30**, the (*S*)-*N,N*-dimethylalanine moiety at C29 could be introduced under either Keck[31] (DCC, DMAP·HCl) or Yamaguchi[26] (TCBC, DMAP, Et_3N)



Scheme 5 Completion of the total synthesis of aplyronines A and D.

esterification conditions. This afforded a product that was configurationally pure with respect to the amino acid stereocentre. Cleavage of all three TES ethers using HF·pyr then afforded aplyronine C (**3** in Figure 1).[32] At this stage, our strategy required the crucial site-selective esterification of the C7 hydroxyl with *N,N,O*-trimethylserine in the presence of the C9 and C25 secondary hydroxyls. After some exploratory investigations,[33] we found that this transformation could be accomplished under Yamaguchi conditions with complete site-selectivity at C7, where careful monitoring by TLC ensured that no *bis*-esterification product at C9 was formed. The use of racemic *N,N,O*-trimethylserine gave rise to a 1:1 mixture of (*S*)- and (*R*)-diastereomeric esters in good approximation to the reported 1.1:1 *dr* in the natural product. To our satisfaction, the direct transformation of aplyronine C into aplyronine A was achieved in 61% yield (82% BRSM), and all the spectroscopic data agreed with that reported for natural aplyronine A.[3a,34] This pleasing result validated our streamlined protecting group strategy, which contrasts with the more elaborate differential protection of the various alcohols employed previously.[5,11]

We now focused on completing the total synthesis of aplyronine D (**2**). By substituting the *N,N*-dimethylalanine ester at C29 for *N,N*-dimethylglycine, an analogous sequence was performed from the pivotal C29 alcohol **30** to afford **2** (43%, 79% BRSM, 3 steps). Gratifyingly, the spectroscopic and biological data obtained for synthetic aplyronine D was an excellent match for that reported for the natural product (copies of NMR spectra were kindly provided by Prof. Yamada).[3c] Notably, this constitutes the first total synthesis of this much rarer, yet apparently more active, aplyronine congener.[34] As in the related case of aplyronines A and C, its presumed biosynthetic precursor with a free hydroxyl at C7 is also likely to be a natural product. We designate this congener as aplyronine I (see the ESI, structure **30a**), although as far as we are aware this has not yet been isolated from extracts of *Aplysia kurodai*.

Finally, in work towards the synthesis of designed analogues suitable for ADC applications, we set out to prepare the linker-modified aplyronine **4** (Scheme 6). Alteration of the actin-binding tail region of the aplyronines at the C29 position has been shown to have little effect on the cytotoxicity.[35] Furthermore, inspection of the 1:1 aplyronine A-actin crystal structure[6] suggested that elaboration of the amino acid moiety at C29 would not be expected to impact on the key ligand-protein binding interactions. Importantly, our modular synthetic route offers the opportunity to introduce a range of functional handles at C29 using the alcohol **30** for late-stage diversification. The initially designed aplyronine analogue **4** contains an Fmoc-protected primary amine for bioconjugation studies, for example through the derived maleimide or functionalised amide.[36]

To construct analogue **4**, attachment of the modified glycine derivative **31** (see the ESI for its preparation) again proceeded smoothly under Yamaguchi esterification conditions (TCBC, DMAP, Et_3N) and the three TES ethers were cleaved on treatment with HF·pyr (91%, 2 steps). The trimethylserine residue was then attached site-selectively at C7 in the resulting triol using a similar procedure to that developed for aplyronines A and D. Furthermore,

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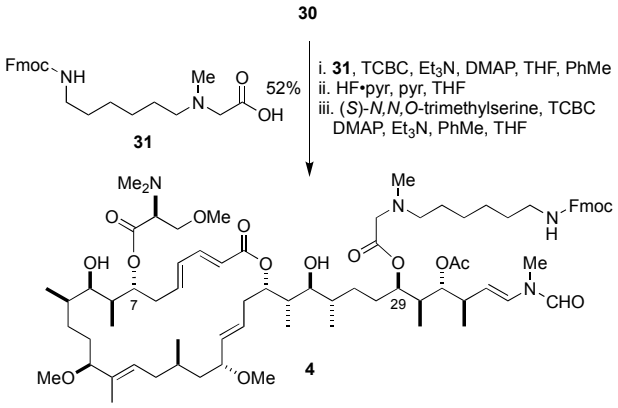
it was demonstrated that this transformation could be achieved stereospecifically using enantiopure (*S*)-*N,N,O*-trimethylserine (see the ESI for its preparation) to afford **4** (58%) as a single diastereomer.[37,38] Given the key structural requirement for the presence of the *N,N,O*-trimethylserine moiety for the potent biological activity of aplyronine A, it is likely that each epimer will have different binding affinities with tubulin and hence variable cytotoxicity, thus the preparation of configurationally pure stereoisomers will allow these SAR effects to be investigated in future work.[39,40]



Scheme 6 Preparation of the C29 linker-modified aplyronine **4**.

Conclusions

In conclusion, we have achieved the total synthesis of the potent antimitotic macrolides aplyronines A (1.3%) and D (1.1%) in 29 steps LLS based on an improved second-generation route that has provided straightforward access to multi-gram quantities of key intermediates. Additionally, we have prepared the linker-modified version **4** for bioconjugation studies in the context of exploring the use of aplyronines as novel cytotoxic payloads for ADCs in targeted cancer chemotherapy. We envisage that this flexible and modular synthetic route can provide a sustainable supply of the aplyronines and their designed analogues for further biological studies, and in future work will adapt it to generate novel aplyronine-based payloads for the discovery of improved ADCs.

Conflicts of Interest

There are no conflicts of interest to declare.

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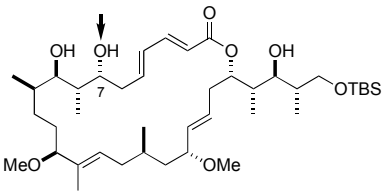
33 Model studies into the esterification site-selectivity were carried out on the truncated macrocyclic triol shown below. These led to the development of suitable reaction conditions for site-specific attachment of *N,N,O*-trimethylserine at the C7 hydroxyl group.



34 Preliminary screening results have confirmed the expected sub-nanomolar antiproliferative activity of our synthetic samples of aplyronines A and D in an extensive panel of human cancer cell lines. Detailed biological evaluation will be reported in due course.

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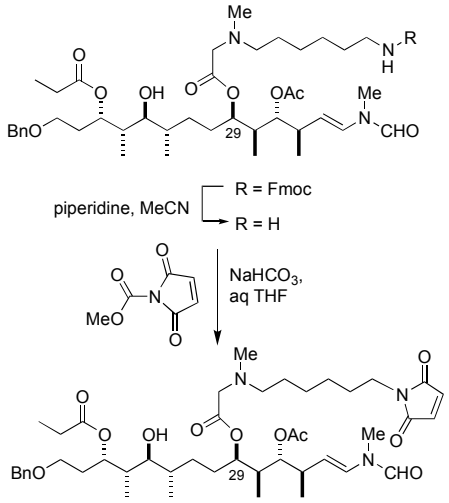
36 Using an extended aplyronine side chain as a model system, we have demonstrated the elaboration of the Fmoc-amino substituted linker into a maleimide-based Michael acceptor for bioconjugation with cysteine residues, as shown below.



37 The biological activity of **4** will be presented elsewhere in the context of ADC development.

38 Yamada (ref 5) and Kigoshi (ref 11) both reported some racemisation of the activated carboxylic acid under their reaction conditions. In our case, the retention of configuration to provide a single diastereomer in the esterified product **4** could be attributed to the lower temperature employed and shorter time required for a less hindered substrate.

39 It is unclear whether the differing epimeric ratios of amino acids present in the aplyronines are a natural feature or an artefact of the isolation procedure. The actual producing organism for the aplyronines is believed to be cyanobacteria, which are ingested from algae consumed by the sea hare in its diet.

40 For related synthetic work from our group, see: I. Paterson and N. Y. S. Lam, J. Antibiot., 2018, doi: 10.1038/ja.2017.111.

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Toward Aplyronine Payloads for Antibody-Drug Conjugates: Total Synthesis of Aplyronines A and D

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We report an expedient total synthesis of aplyronines A and D, together with a linker-modified analogue for bioconjugation studies.