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Stereoselective synthesis of allele-specific BET inhibitors†

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Developing stereoselective synthetic routes that are efficient and cost-effective allows easy access to biologically active molecules. Our previous syntheses of allele-selective bumped inhibitors of the Bromo and Extra-Terminal (BET) domain proteins, Brd2, Brd3, Brd4 and BrdT, required a wasteful, late-stage alkylation step and expensive chiral separation. To circumvent these limitations, we developed a route based on stereocontrolled alkylation of an N-Pf protected aspartic acid derivative that was used in a divergent, racemisation-free protocol to yield structurally diverse and enantiopure triazolodiazepines. With this approach, we synthesized bumped thienodiazepine-based BET inhibitor, ET-JQ1-OMe, in five steps and 99% ee without the need for chiral chromatography. Exquisite selectivity of ET-JQ1-OMe for Leu-Ala and Leu-Val mutants over wild-type bromodomain was established by isothermal titration calorimetry and X-ray crystallography. Our new approach provides unambiguous chemical evidence for the absolute stereochemistry of the active, allele-specific BET inhibitors and a viable route that will open wider access to this compound class.

Chemical biology and therapeutic development rely on the design or discovery of biologically active compounds that typically contain one or more stereocenters. Amongst the different stereoisomers for a given compound, it is often the case that only one (so-called eutomer) exhibits the desired biological activity, while the other(s) (distomers) may be inactive or have toxic and off-target effects.[1] Studying and testing diastereomeric or racemic mixtures has limitations and could lead to unwanted or artefactual results, it is therefore important to develop stereoselective routes and processes which yield solely the desired biologically active molecule.[2–5]

The four Bromo and Extra-Terminal (BET) proteins, Brd2, Brd3, Brd4 and BrdT, play a crucial role in transcriptional regulation and other processes such as cell proliferation and cell cycle progression.[6–8] BET proteins have become attractive therapeutic targets as their misregulation has been linked to diseases such as cancer, neurological disorders and inflammation.[9,10] Association to disease has fuelled great interest in the field to develop small molecule BET inhibitors, many of which are in the clinic.[11–14] Many BET inhibitors include a triazolodiazepine scaffold, including JQ1 (ref. 15) and I-BET762 (ref. 16) (Fig. 1). Due to the high conservation of BET bromodomains at the acetyl-lysine binding pocket, these inhibitors are pan-selective so do not significantly discriminate between the bromodomains within and across the BET

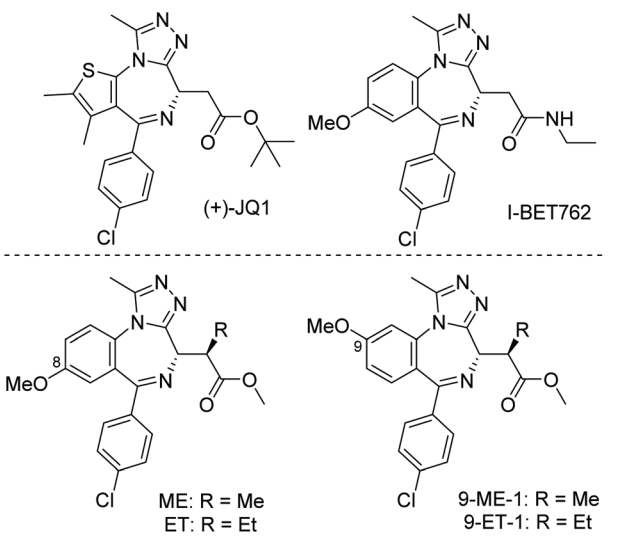

Fig. 1 Pan and allele-selective BET inhibitors. Pan-selective inhibitors (+)-JQ1 and I-BET762 (top) and allele selective probes ME, ET, 9-ME-1 and 9-ET-1 (bottom).

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†Electronic supplementary information (ESI) available: Supplementary results (Fig. S1–S5 and Table S1); Materials and methods section and Scheme S1; supplementary references and NMR spectra. Atomic coordinates and structure factors for the crystal structure of Brd2(BD2)-L383V mutant in complex with ET-JQ1-OMe has been deposited in the protein databank (PDB) under accession number 6YTM. See DOI: 10.1039/d0ob01165g

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family.[17,18] More recently, compounds have been reported to show selective binding to the first bromodomains (BD1), or to the second bromodomains (BD2), of the BET family.[19–22] Although these compounds can discriminate between BD1 and BD2 within a given BET protein, they still cannot discriminate across the four BET proteins.

To aid individual intra-BET selectivity, we previously developed a chemical genetics approach to engineer orthogonal protein/ligand pairs between the BET proteins and selective inhibitors.[23] Our "bump & hole" approach involved the introduction of a single point mutation to the target BET bromodomain by replacing a leucine residue that is conserved across all BET bromodomains, with a smaller residue (*e.g.* alanine) to generate a "hole".[24] Simultaneously, an alkyl "bump" is incorporated at a diastereotopic, methylene group on the parent scaffold based on the I-BET762 inhibitor, aimed to both complement the size of the "hole" and provide a steric clash to the wild-type protein. This approach led to the generation of allele specific chemical probes ET (ref. 24) and 9-ME-1 (ref. 25) (Fig. 1) targeting the leucine to alanine (L/A) or the less disruptive, leucine to valine (L/V) mutation, respectively. We used this new system to dissect individual roles of BD1 *vs.* BD2 in Brd4 and the other BET proteins and showed that while the BD1 is necessary and sufficient for chromatin binding, the BD2 plays a role in transcriptional regulation.[24,25]

Our previous approaches for incorporation of an alkyl "bump" into the I-BET scaffold involved alkylation of a potassium enolate to afford bumped I-BET derivatives with undesired diastereoselectivity and in low yields (Fig. 2A). Epimerisation was required to enhance the amount of desired diastereomer, and further separation by high performance liquid chromatography (HPLC) to isolate the desired isomer. Due to the use of potassium hexamethyldisilazide (KHMDS) for the enolization, and the need of an epimerisation step at such a late stage of the synthesis, the products are racemic and require chiral separation to isolate the eutomers.[24–26] Separation of the enantiomers can be costly (up to £1000 for <150 mg of racemate) and leads to loss of material during the separation.

We sought to address these problems by developing a new stereoselective synthetic strategy. We hypothesised that incorporating the "bump" earlier in the synthesis *via* a diastereoselective alkylation of an aspartic acid derivative would circumvent the limitations of the original route (Fig. 2B). Here, we describe a new synthetic route that allows the preparation of both novel and previously described bumped BET inhibitors stereoselectively in 99% ee. We also provide unambiguous chemical evidence to the absolute stereochemistry of the active allele-specific ligand, previously only assumed based on co-crystal structures.[25]

Our first efforts to alkylate (+)-JQ1 directly proved unsuccessful, likely due to the steric hindrance caused by the *tert*-butyl ester. Conversion from the *tert*-butyl to a methyl ester was required to allow for the introduction of the bump. Alkylation with KHMDS and alkyl iodides proceeded with undesired diastereoselectivity towards the (*S*,*S*) diastereomer over the (*S*,*R*) diastereomer in overall alkylation yields of approx. 20%. Epimerisation of the major (*S*,*S*) isomer with sodium methoxide allows access to the desired (*S*,*R*) isomer in a 1 : 1 ratio with the starting (*S*,*S*) isomer. However, the use of strong bases during alkylation and additional epimerisation steps may lead to complete racemisation of both stereocentres as previously reported,[24–26] (see Scheme S1 in ESI†). With enantiopure (+)-JQ1 costing >$750 per gram, and its derivatives *e.g.* (+)-JQ1 carboxylic acid being even more expensive, this wasteful approach is not a viable strategy for the preparation of enantiopure bumped JQ1.

To efficiently prepare the desired bumped JQ1 derivatives as single enantiomers, we sought to stereoselectively introduce the "bump" earlier in the synthesis. Our synthetic methodology was based on previously reported stereoselective alkylation of L-aspartic acid diester derivatives.[27–30] We so hypothesized that protection of the amino group with both 9-phenyl-9-fluorenyl (Pf) and benzyl (Bn) groups could drive diastereoselective alkylation, with the Pf group providing strong steric hindrance of the β-carbon to the nitrogen while also protecting the α-proton from epimerisation.

L-Aspartic acid derived diester **1** was first treated with benzaldehyde in dichloromethane (DCM) and the formation of the intermediate imine was monitored by ^{1}H-NMR. Reduction of the imine with sodium borohydride yielded the mono-benzyl protected amine **2**. Amine **2** was treated with 9-phenyl-9-fluorenyl bromide, lead(II) nitrate and tribasic potassium phosphate in acetonitrile to form the *N*-diprotected diester **3**. Deprotonation of diester **3** with lithium hexamethyldisilazide (LHMDS) at −78 °C in tetrahydrofuran (THF) afforded the desired *E*-lithium enolate, which was reacted with methyl iodide at −40 °C over 16 h. This yielded methylated diastereomers **4a** (*S*,*R*) and **4b** (*S*,*S*) in a 6 : 1 ratio respectively. Ethylated compounds **5a** and **5b** were prepared in a similar way by deprotonation of diester **3** with LHMDS at −78 °C in THF fol-

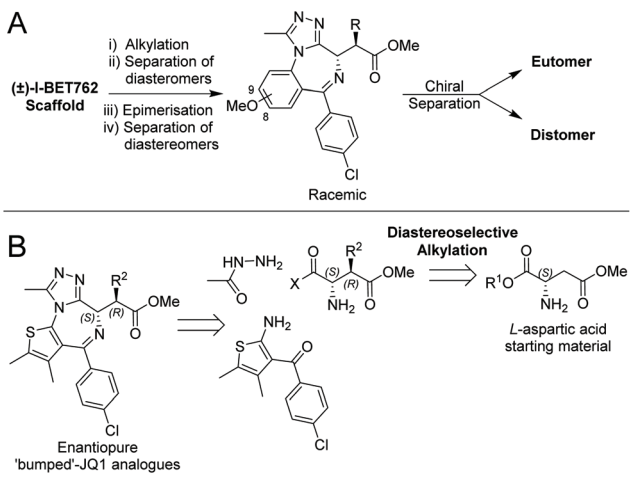


Fig. 2 Synthetic routes to bumped BET inhibitors. (A) Previous synthetic strategy for bumped I-BET762 derivatives. (B) Retrosynthetic analysis for enantiopure bumped JQ1 derivatives.

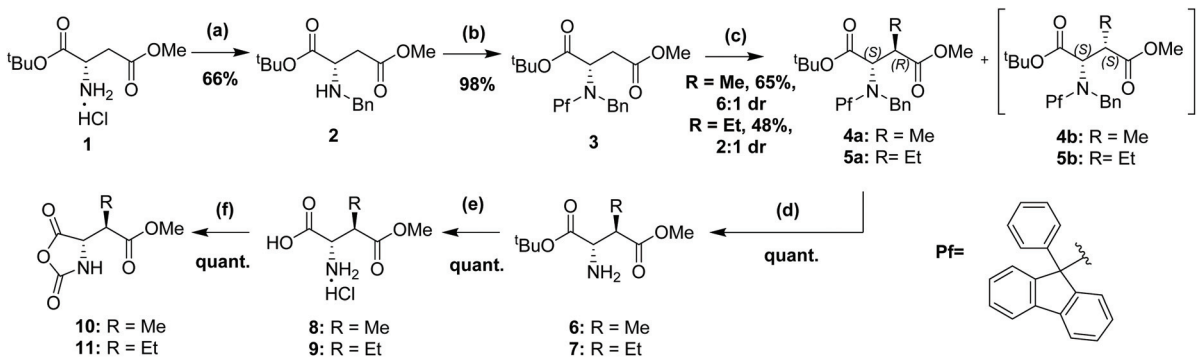
lowed by addition of ethyl iodide and stirring at −78 °C for 16 h. This was left for a further 24 h at −23 °C to yield diastereomers **5a** (*S*,*R*) and **5b** (*S*,*S*) in a 2 : 1 ratio respectively. The choice of LHMDS over the respective potassium base, KHMDS, was motivated by prior findings that switching between these bases can reverse the diastereoselectivity on a similar aspartate derived diester to compound **3**.[27] Using the potassium base leads to a chelate controlled enolate-ester intermediate which has the opposite geometry to the non-chelated, 'open' lithium enolate intermediate and influences facial selectivity to attack by the electrophile (alkyl iodide).

Removal of both Pf and Bn groups were performed by hydrogenation of alkylated diesters **4a** and **5a** with a suspension of 10% palladium on carbon (Pd/C) in acetic acid to give free amines **6** and **7** in high yields. The resulting free amines were dissolved in a 1 : 1 mixture of trifluoroacetic acid (TFA) and dichloromethane to achieve the *tert*-butyl ester deprotection and leave the free amino acid as a TFA salt. The TFA salts were then dissolved in 2 M HCl and freeze-dried to obtain the amino acids **8** and **9** as HCl salts. Conversion of salts proved crucial for the next step as any TFA present led to the formation of trifluoroacetamide by-products. Amino acids **8** and **9** (HCl salts) were treated with triphosgene in THF over 16 h to yield the key *N*-carboxyanhydrides (NCAs) **10** and **11**, as precursors of the alkylated sidechain fragment. These were used in the next steps without the need for further purification (Scheme 1).

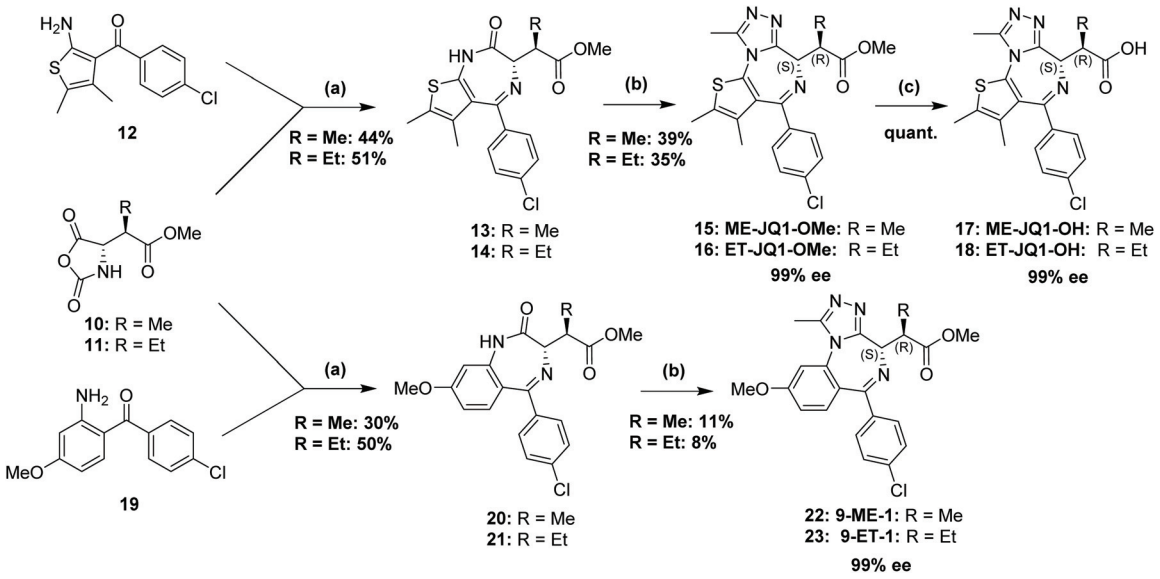
Next, thienodiazepines **13** and **14** were formed in a condensation reaction between NCAs **10** and **11** and amino ketone **12** by heating in the presence of TFA and subsequently triethylamine (TEA) in toluene as reported by Fier *et al.*[31] The use of this methodology for the benzodiazepine ring formation was crucial in our synthetic strategy as it was found to retain the stereochemistry of the amino acid derived NCA. Deprotonation of the lactam in both **13** and **14** with potassium *tert*-butoxide and addition of diethyl chlorophosphate, as described,[32] gave the activated phosphorylimidate intermediate which was not isolated. This was subsequently reacted with acetylhydrazine which led to the formation of the triazole ring in the final compounds **15** (ME-JQ1-OMe) and **16** (ET-JQ1-OMe) with 99% ee determined with supercritical fluid chiral chromatography (Scheme 2, see ESI† for analytical details). Overall, we were able to achieve ∼40 mg of enantiopure product in just five, yield limiting, steps from <£100 worth of starting materials. In comparison, our previous approach required six steps, including expensive chiral purification (∼£1000) and 1 g of JQ1 ($750) to achieve the same amount of pure product.

Quantitative conversion to carboxylic acids **17** (ME-JQ1-OH) and **18** (ET-JQ1-OH) was achieved by treating esters **15** and **16** in a 4 : 1 mixture of THF to either a 0.54 M or 0.65 M lithium hydroxide (LiOH) solution respectfully. Heating to 45 °C was required for ethyl bumped compound **16** due to the conversion being much slower in comparison to the methyl bumped compound **15**. These very mild conditions were essential to avoid epimerisation of the alkylated stereocenter adjacent to the carbonyl group. Using higher concentrations of LiOH and/or higher temperatures resulted in an increased rate of hydrolysis but led to substantial epimerisation to the undesired (*S*,*S*) diastereomer. Access to these free acids allows the possibility for further functionalisation (*e.g. via* amide or ester bond formation).

Having achieved the novel bumped JQ1 derivatives, we next sought to demonstrate the versatility and scope of our new route by attempting to synthesise the I-BET-based bumped probes, 9-ME-1 and 9-ET-1.[25] By using NCA precursors **10** and **11**, and treating them with aminobenzophenone **19** in the same condensation reaction as described previously, yielded benzodiazepines **20** and **21**. Subsequent triazole ring formation *via* a similar phosphorylimidate intermediate as described above yielded the final ligands, **22** (9-ME-1) and **23** (9-ET-1), with 99% ee determined with supercritical fluid chiral chromatography (see ESI† for analytical details). We have also demonstrated the accessibility for the (*S*,*S*) diastereomer (reported as **16*** in the ESI†) of **16** using the minor ethylated diastereomer **5b** as the starting point. Stereoselective access to 'inactive' stereoisomers provides important negative



Scheme 1 Stereoselective synthesis of *N*-carboxyanhydrides **10** and **11**. Conditions: (a) (i) Na_2CO_3, EtOAc, H_2O, (ii) PhCHO, DCM, 2 h, r.t., (iii) $NaBH_4$, MeOH, 1 h, 0 °C–r.t. (66%); (b) PfBr, K_3PO_4, $Pb(NO_3)_2$, MeCN, r.t., 4 h (98%); (c) (i) LHMDS, THF, −78 °C, 1 h, (ii) MeI/EtI, −78 °C to −23 °C, 48 h/96 h (65%/48%); (d) H_2, Pd/C, AcOH, r.t., 24 h (quant.); (e) (i) TFA, DCM, r.t., 2 h, (ii) 2 M HCl, freeze dry (quant.); (f) triphosgene, THF, r.t., 16 h (quant.).



Scheme 2 Formation of thienodiazepines **15–18** and benzodiazepine derivatives **22** and **23**. Conditions: (a) (i) TFA, toluene, 60 °C, 0.5 h, (ii) TEA, 80 °C, 16 h (30–51%); (b) (i) KO^{t}Bu, THF, −78 °C to −10 °C, 0.5 h, (ii) $(OEt)_2P(O)Cl$, −78 °C to −10 °C, 0.75 h, (iii) $AcNHNH_2$, r.t., 1 h, (iv) *n*-butanol, 90 °C, 1 h (8–39%); (c) LiOH, THF : H_2O 4 : 1, R = Me; r.t., 1 to 3 days; R = Et: 45 °C, 1 week (quant.).

controls to increase validity and robustness of findings in both biophysical and biological assays.

To further characterise our novel bumped compound, we studied the binding of **16** to Brd4(2) L387A, L387V and wild-type using isothermal titration calorimetry (ITC). We found undetectable/no binding of **16** to the wild-type protein (Fig. 3A, see also ESI Fig. S5†), consistent with ethyl bumped compounds reported previously.[24,25] Crucially, **16** demonstrated very high binding affinity to both L/A and L/V mutants with equipotent K_d values of 65 nM (Fig. 3A, see also ESI Fig. S3 and S4†). To validate the binding mode, we solved a high resolution (1.56 Å) X-ray structure of Brd2(2)^{L383V} co-crystallised with **16** (Fig. 3B, see also ESI Fig. S2 and Table S1†). We found that **16** adopts a similar binding mode to 9-ME-1 and 9-ET-1 (see ESI Fig. S1†), positioning the ethyl "bump" towards the "hole" formed by the L/V mutation.

In summary, we describe a versatile stereoselective approach to successfully synthesise bumped BET inhibitors. We demonstrate scope by synthesizing both novel JQ1 derivatives and previously described I-BET762 derivatives, all in 99% ee. Compared to the previous method, our new route achieves enantiopure products in one less step, from widely available and relatively inexpensive starting materials, while avoiding wasteful, late-stage alkylation steps and chiral separation. We qualified the remarkable allele-selectivity of ET-JQ1-OMe over wild-type and provided unambiguous chemical evidence to the absolute stereochemistry of the eutomer. Access to carboxylic acid derivatives retaining enantiomeric purity enables functionalisation into conjugates such as biotinylated and fluorescent probes and PROTACs,[23] which will further expand the scope and utility of this new synthetic strategy for chemical biology investigation.

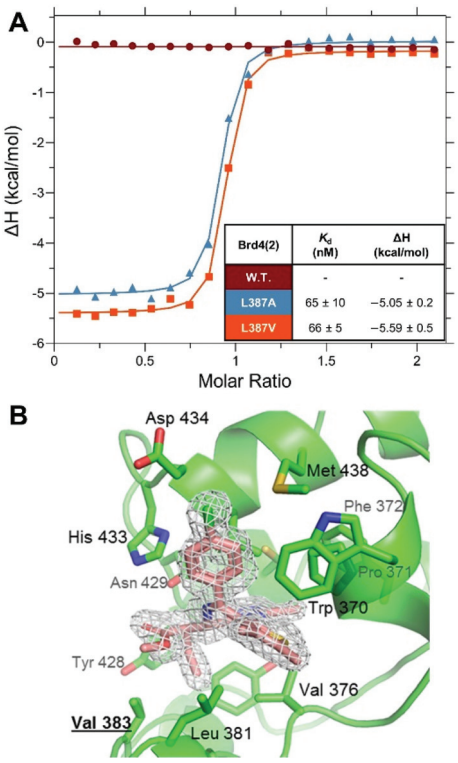


Fig. 3 Biophysical and structural characterization of novel JQ1-based bumped inhibitor. (A) ITC titrations of ET-JQ1-OMe (**16**) against wild-type (W.T.) (maroon), L387A (blue) and L387V (red) constructs of Brd4(2). Table shows values taken as a mean and standard deviation from three replicates. (B) Co-crystal structure of **16** (pink carbons) with Fo-Fc omit map (grey mesh, contour: 3σ) bound to Brd2(2)^{L383V} mutant (green, cartoon representation; green carbons, binding site side chains) (PDB code: 6YTM). Bump and hole residue **Val 383** is highlighted.

Abbreviations

$AcNHNH_2$	Acetyl hydrazine
AcOH	Acetic acid
BD1	First bromodomain
BD2	Second bromodomain
BET	Bromo and extra-terminal
Brd	Bromodomain
DCM	Dichloromethane
ITC	Isothermal titration calorimetry
KHMDS	Potassium hexamethyldisilazide
LHMDS	Lithium hexamethyldisilazide
NCA	*N*-Carboxyanhydride
PfBr	Phenylfluorenyl bromide
PROTACs	Proteolysis targeting chimeras
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran

Author contributions

The manuscript was written through contributions of all authors, and all authors have given approval to the final version of the manuscript.

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Conflicts of interest

The authors declare the following competing financial interest (s): The Ciulli laboratory receives or has received sponsored research support from Amphista Therapeutics, Boehringer Ingelheim, Eisai, Nurix, and Ono Pharmaceuticals.

A.C. is a scientific founder, shareholder, non-executive director and consultant of Amphista Therapeutics, a company that is developing targeted protein degradation therapeutic platforms. The remaining authors report no competing interests.

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