Simple, Enantiocontrolled Azetidine Library Synthesis via Strain Release Functionalization of 1-Azabicyclobutanes

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ABSTRACT: A simple, modular, programmable approach to access complex stereopure azetidines through strain-release functionalization is disclosed. The synthetic methods developed enable parallel synthesis of stereodefined azetidines that would be otherwise laborious to produce. Given the privileged nature of these structures, a set of stereoprobes for use in activity-based protein profiling was prepared and evaluated, revealing proteins in human cancer cells with were liganded with clear stereo- and chemo-selectivity.

The azetidine (azacyclobutane) heterocycle, a four-membered saturated cyclic amine, is found in various natural and synthetic compounds with unique biological activities. The azetidine's physicochemical properties make it an increasingly popular scaffold in drug discovery despite being known since the 1950's.[1] Azetidines can be used as bioisosteric replacements for more common saturated heterocycles such as piperazines, piperidines, pyrrolidines, leading to improved drug-like properties including lipophilicity, solubility, and *in vitro* metabolism.[2] Indeed, numerous approved drugs such as delafloxacin, azelnidipine, tebanicline, baricitinib, cobimetinib, tebipenem, and siponimod feature an azetidine. Increasingly complex substituted azetidines, such as ones containing 2,3-disubstitution, have become commonplace (Figure 1A).[3] Azetidines have also prominently featured in the context of chemical proteomic studies.[4] Notwithstanding the relevance of azetidines in drug discovery and recent innovation in synthetic methodology, a need remains for new methods of azetidine construction that allow access to expanded chemical space, ideally in an expeditious fashion. Indeed, current approaches to synthesizing more complex azetidines in enantiopure form either employ multistep ring-synthesis strategies (which are not amenable to library synthesis) or C–H functionalization of a preformed azetidine, which is inherently limited to arylation (Figure 1B).[5] Disclosed herein is a different approach for rapid, modular synthesis of enantio- and diastereopure azetidines that capitalizes on simple strain release functionalization of pre-formed 1-azabicyclobutanes (ABBs).[5d,6] The developed chemistry can be employed to generate libraries of diverse optically active azetidines. Chemical proteomic profiling of stereoisomeric sets of cysteine-reactive acrylamides derived from the new azetidine library reveals ligandable protein sites distinct from those of azetidine probes synthesized using C–H arylation. These findings illustrate the potential for the method of azetidine synthesis reported herein to facilitate the discovery of chemical probes and drug leads.

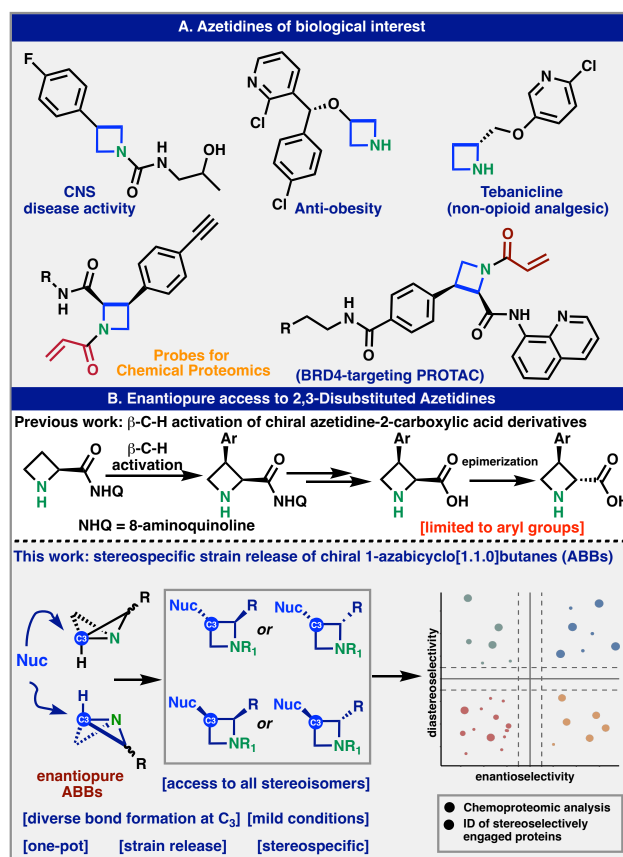

Figure 1. (A) Azetidines are features prominently in numerous pharmaceuticals and probes in chemical biology. (B) the described work overcomes limitations in stereoselective azetidine synthesis; when elaborated with acrylamide substitution they prove useful in chemical proteomics studies.

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As the number of substituents increases on the azetidine ring, simplified and modular access can be challenging. Indeed, the common route to such scaffolds involves cyclization from acyclic precursors already bearing terminal substitution, which in turn require multistep routes for preparation. An alternative approach to modularly access 2,3-disubstitution relies on C–H activation approaches (Figure 1B) but so far these are limited to (hetero)arene substitution at C–3 followed by directing group cleavage.[5a, 5c] That approach delivers cis-2,3 substitution and if the trans-stereochemistry is desired an additional epimerization step is required. The Didier group reported an innovative approach to access cis-2,3 substitution utilizing azetinyl-carboxylic acid followed by asymmetric reduction.[5b] Previously, our group reported a general strategy for the direct functionalization of strained C–C and C–N bonds, including that of azabicyclo[1.1.0]butanes (ABBs).[6b] Since then, numerous reports have appeared on both the polar and radical functionalization of ABBs.[5d]

Based on early literature findings showing that ABBs could undergo diastereospecific opening, it was hypothesized that enantiopure 2-substituted ABBs may be amenable to stereospecific openings (strain-release) with a variety of nucleophiles.[7] These ABBs were thus targeted for synthesis to explore their downstream functionalization with nucleophiles of varying pKas. For the purposes of this study, a suitably protected 2-hydroxymethyl substituent was chosen (*vide infra*). With this goal in mind, a scalable, enantiocontrolled approach to procure all four diastereomers of 3,4-dibromo-1-(*tert*-butoxy)butan-2-amine (ABB precursor) was developed (Figure 2A). The simple four-step sequence commencing from 2-tert-butoxy-ethanol (ca. \$0.25/g) involved: (1) oxidation to form the corresponding aldehyde (IBX), (2) reaction with either (R) or (S) Ellman's sulfinamides to afford the enantiopure *tert*-butanesulfinyl imines, (3) stereoselective 1,2-addition with vinylmagnesium bromide, and (4) addition of Br_2 followed by column chromatography to afford all the corresponding chiral ABB precursors. An alternative approach to access these ABB precursors using asymmetric π-allyl substitution was also developed (See SI for details).

Stable chiral building blocks **2a-2d** smoothly undergo sequential ring-closing reactions upon treatment with MeLi (Et_2O, -78 °C) to afford enantiopure ABBs **3a-3d,** which can be isolated in high purity by simple extraction (Figure 2A). These ABBs have been found to be stable for long term storage under inert and basic conditions (see SI for details).

To set the stage for azetidine library synthesis, a variety of nucleophiles and conditions were screened and optimized conditions were developed (Figure 2B). When exposed to acidic conditions the ABBs were found to be unstable and dimerization was observed. In the literature, exposure of ABBs to weak acid leads to incorporation of the nucleophilic counterion at the C-3 position (e.g. AcOH).[8] With this data in mind, two sets of conditions were employed depending on the pKa of the nucleophile: (1) acidic nucleophiles e.g. carboxylic acids, phenols, thiophenols, and phosphates were added in directly (THF at rt) or (2) basic nucleophiles such as anilines, alcohols, and alkyl amines added in smoothly with the addition of TfOH (0.9-1 equiv, CH_3CN or toluene, -78 to -40 °C followed by stirring at rt). TfOH was chosen due to its non-nucleophilic conjugate base. In accord with prior studies, strong nucleophiles (Grignard reagents) could also be added stereospecifically to generate C–C linkages at C-3.[9] Finally, by utilizing both the acidity of C-3 and the basicity of the amine, a C-3 double functionalization could be achieved as a result of sequential deprotonative functionalization followed by acid-catalyzed strain-release opening.

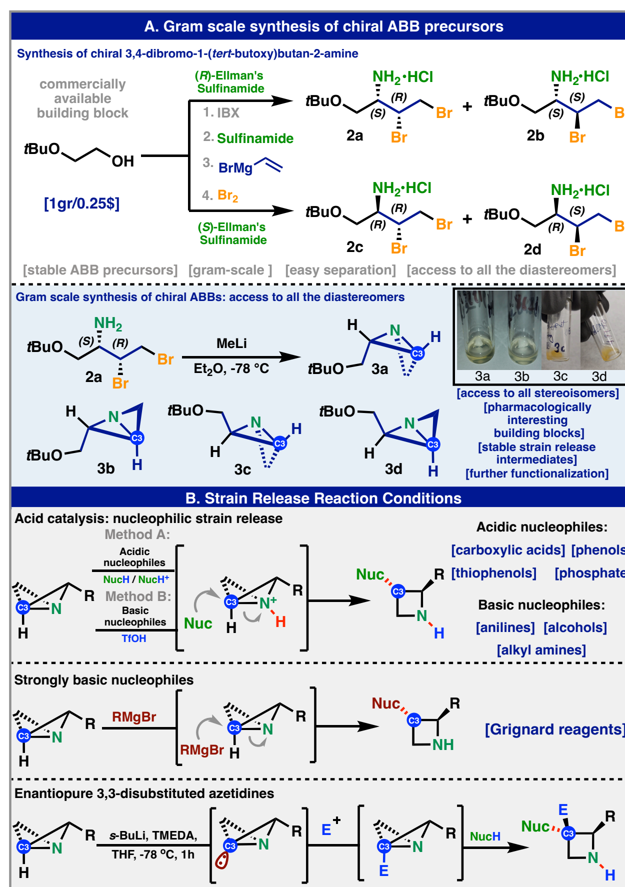

Figure 2. (A) Scalable synthesis of ABB precursors and their smooth conversion to stable enantiopure ABBs. (B) development of conditions for their downstream functionalization.

With the optimized set of conditions (Methods A and B) in hand, the scope of this method was investigated as outlined in Table 1. Numerous nucleophiles were enlisted such as amines, alcohols, thiols, carboxylic acids, and phosphates. Acidic and basic nucleophiles as well as Grignard reagents, amino acid functionalization and double C3 functionalization were successfully demonstrated. An electrophilic quenching agent (Boc anhydride) was utilized to simplify purification. The products were obtained in 36–87% yields with excellent diastereoselectivities (>20:1), on scales ranging from 0.1 to 0.8 mmol. The stereochemical outcomes of these reactions were verified by X-ray crystallography (**2b', 2d, 13**-trans, **18**-cis, **18**-trans, **21**-trans, and **28'**). Thus, when the starting material carries an acidic proton, Method A enabled ring opening using phenols (**14** and **26**), thiophenol (**20**) carboxylic acids (**19** and **25**), and phosphate (**10**). Using Method B, basic or neutral e.g. amines (**8**, **22**, and **23**),

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anilines (**6** and **7**) and alcohols (**11-12**) led to smooth ring opening. Despite the fact that TfOH is a strong acid, the chemoselectivity and functional-group tolerance was high, enabling use of acid-sensitive groups such as tert-butyl ethers and esters. Additionally, select previously reported nucleophiles were found to successfully undergo C-3 strain release nucleophilic substitution, for example Grignard reagents (**15**, **16** and **17**)[9] and azides (**9**).[10] Finally, stereospecific double functionalization at the C3 position was investigated. This was achieved by treating the ABB (**3b-c)** with *t*BuLi to deprotonate the C3 site, resulting in ABB lithium (ABB-Li).[10b, c]

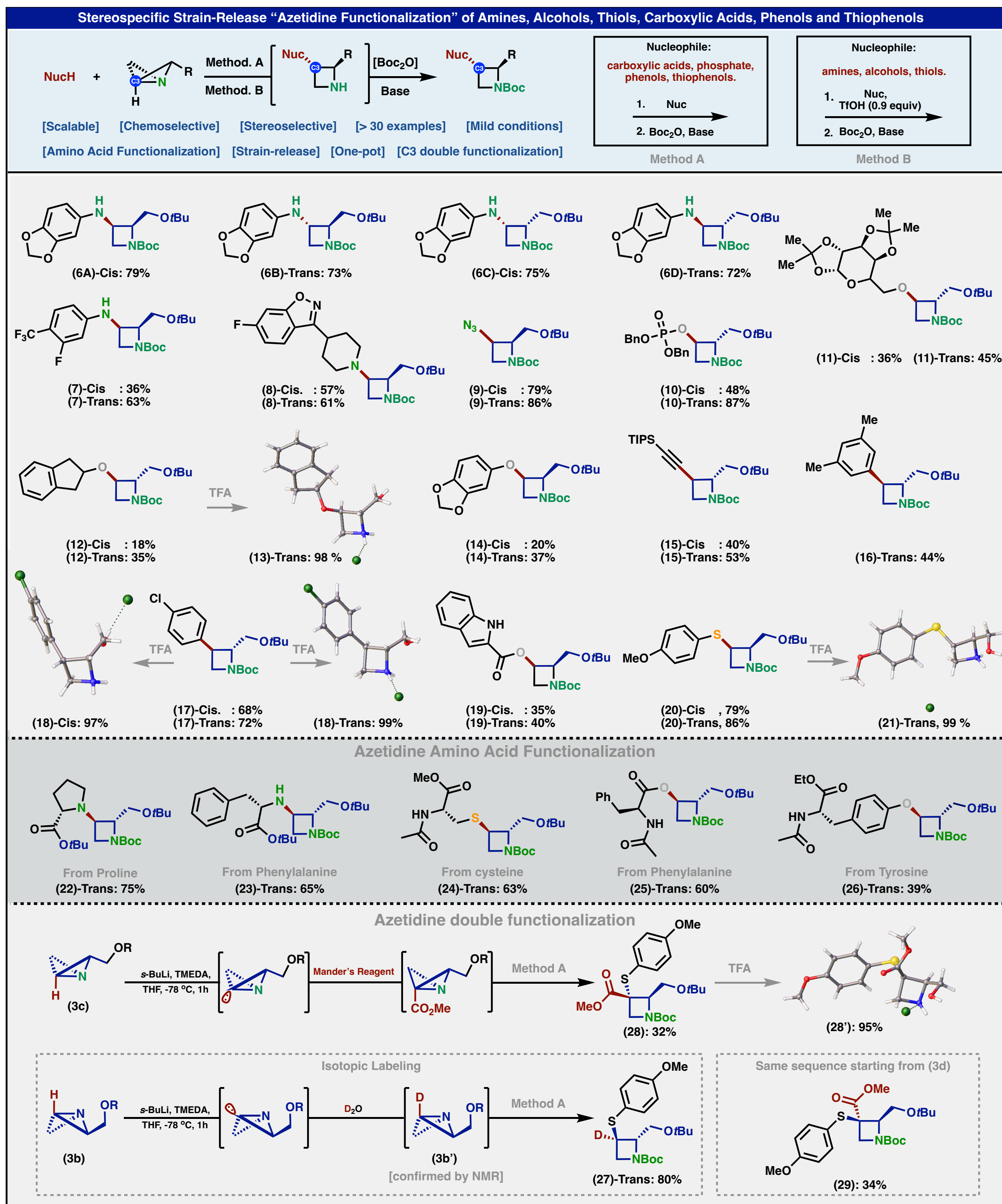

Table 1. Scope of stereospecific strain release opening of enantiopure ABBs. All products were obtained with dr >20:1.

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This species was then quenched with D_2O to give a C3 deuterium labeled ABB, which was further reacted with 4-methoxythiophenol (method A) to afford compound **27**. Similar sequential deprotonative functionalization was utilized to form compounds **28** and **29** by using methyl cyanoformate as the electrophile (see SI for support for stereochemical outcome).

The products of these ring-opening reactions feature multiple functional handles for further elaboration, including the 2-(hydroxymethyl) and amine substituents. We aimed to showcase the utility of this elaboration potential by synthesizing a new class of cysteine-reactive, stereopure 2,3-disubstituted azetidine acrylamides. Such azetidine "stereoprobes" can identify ligandable cysteines on proteins in native cellular environments using the chemical proteomic method activity-based protein profiling (ABPP).[4] Such ligandable cysteines are defined as showing stereoselective reactivity with the azetidine acrylamide stereoprobes, which provides evidence of specific interactions between the small molecules and proteins in cells. Current routes to azetidine stereoprobes use C–H arylation reactions to functionalize enantioenriched azetidine-2-carboxylic acid derivatives,[5a,c] delivering azetidine acrylamides featuring exclusively 3-aryl substitution (e.g. MY-11/12 and MY-1/3, referred to hereafter as 3-aryl stereoprobes or the 3-aryl chemotype).[4b,c,d] We hypothesized that ABPP experiments performed with a structurally distinct set of azetidine acrylamide stereoprobes generated using the newly developed synthetic approach may identify additional ligandable cysteines in the human proteome. Stereoprobe synthesis commenced with nucleophilic opening of enantio- and diastereopure ABBs with an aniline derivative. The C2-carbinol was deprotected and functionalized via SnAR chemistry, and the product of this reaction was subjected to sequential amide coupling reactions to install a pentynyl or methyl amide and an acrylamide on the anilino and azetidino nitrogens, respectively. A total of eight "3-amino stereoprobes" were generated in this fashion, four of which contain an alkyne group (**36A–D**, termed alkyne probes) and four of which contain no alkyne (**37A–D**, termed competitor probes). Each set of four includes all four unique stereochemical permutations.

We performed initial gel-ABPP experiments to benchmark the proteome-wide reactivity of the alkynylated 3-amino stereoprobes 36A–D in comparison to the previously described 3-aryl stereoprobes **MY-11A/B** and **MY-12A/B** (Figure 3A).[4a,c] Exposure of the Ramos human B-cell cancer line to alkynylated stereoprobes (20 or 50 μM, 1 or 3 h), followed by lysis and copper-catalyzed azide–alkyne cyclo- addition (CuAAC)[11] conjugation of stereoprobe-labeled proteins to an azide–rhodamine reporter tag and in-gel fluorescence scanning, revealed that **36A–D** displayed much lower overall proteomic reactivity compared to **MY-11A/B//12A/B** (Figure 3B). Several stereoselective protein interactions were also observed for the 3-amino stereoprobes at both test concentrations and time points of incubation (marked with red asterisks for the 20 μM and 50 μM, 3 h condition in Figure 4B). Based on these initial gel-ABPP data, we selected concentrations of 10 μM and 20 μM, respectively, to evaluate alkyne and competitor 3-amino stereoprobes by protein-directed mass spectrometry (MS)-ABPP.[12]

We performed protein-directed ABPP experiments by treating Ramos cells with non-alkyne competitor stereoprobes (20 μM; **37A–D**) or dimethyl sulfoxide (DMSO) for 2 h, followed by treatment with the corresponding alkyne stereoprobes (10 μM; **36A–D**) for 1 h. Cells were then lysed and stereoprobe-reactive proteins conjugated to biotin-azide by CuAAC, isolated with streptavidin beads, digested with trypsin, labelled with tandem mass tags (TMT), and identified (MS1/MS2 analysis) and quantified (MS3 analysis) by multiplexed (TMT16plex) MS-based proteomics (Figure 4A). Proteins showing greater than threefold enrichment by one stereoprobe compared to its enantiomer were assigned as stereoselectively enriched proteins. These proteins were further designated as being stereoselectively liganded if pretreatment by the corresponding competitor probe resulted in >50% blockade of stereoselective enrichment as compared to pretreatment with DMSO.

The protein enrichment profiles of stereoprobe can be visualized using quadrant plots (Figure 4B), where the positions of proteins on the x and y axes reflect enantioselective and diastereoselective enrichment, respectively, and the size of the dot represents the degree of competitive blockade of this enrichment by the corresponding non-alkyne stereoprobe.[12] In total, 118 proteins were quantified as being stereoselectively enriched by 3-amino stereoprobes, with 20 of these proteins being stereoselectively liganded by competitors (Figure 4C). The 3-amino stereoprobes stereoselectively enrich an overlapping but mostly distinct set of proteins in comparison to 3-aryl stereoprobes (Figure 4D), pointing to the impact of varied functional groups around the azetidine core in impacting stereoprobe binding and reactivity with distinct protein pockets. The 3-amino and 3-aryl stereoprobes also differed in their respective degrees of stereoselective liganding by competitors, in that far fewer proteins were liganded by the 3-amino stereoprobes (Figure 4D). This outcome is potentially consistent with the much lower proteomic reactivity of the 3-amino stereoprobes observed by gel-ABPP (Figure 3B).

The stereoselectively liganded targets of 3-amino stereoprobes span a range of protein classes including nucleotide exchange factors (DENND2D), methyltransferases (TRMT61A) and other proteins responsible for RNA modification (MTO1) and transport (CLUH), scaffolding subunits of metabolic enzymes (UQCRC1), glutathione S-transferases (GSTO1), and kinases (IKBKB, STK39) (see SI Figure S13 for full list). In Figure 4E–G, we highlight the stereoselective enrichment of UQCRC1 by 36B and MTO1 and IKBKB by **36D**, and blockade of enrichment by **37B** and **37D**, respectively. None of these proteins showed strong evidence of stereoselective enrichment by the 3-aryl stereoprobes. In summary, in comparison to previously described 3-aryl-azetidine acrylamides, the 3-amino-azetidine acrylamides displayed lower overall proteomic reactivity that nonetheless included discrete and unique stereoselective liganding events, highlighting the value of gaining synthetic access to structurally diverse azetidine stereoprobes.

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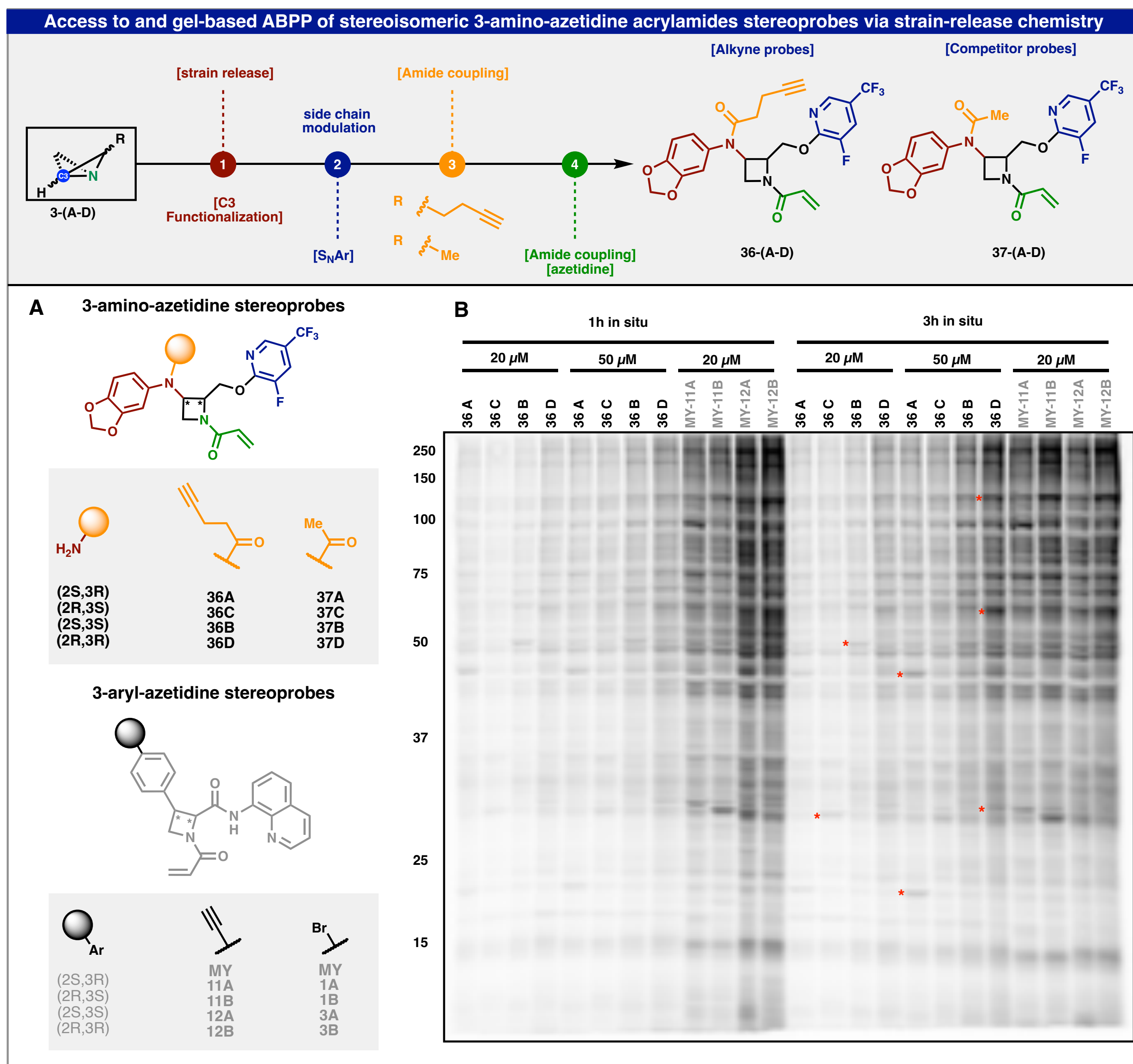

Figure 3. Access to and gel-based ABPP of stereoisomeric 3-amino-azetidine acrylamides stereoprobes via strain-release chemistry. (A) Structure of 3-amino-azetidine acrylamides **36** and **37** in comparison to 3-aryl-azetidine acrylamides **MY-11/12** and **MY-1/3**. (B) Gel-ABPP data for Ramos cells treated with alkyne stereoprobes (20 or 50 μM, 1 or 3 h). Stereoprobe-reactive proteins were visualized by CuAAC conjugation to an azide–rhodamine reporter group, SDS-PAGE, and by in-gel fluorescence scanning. Red asterisks mark representative proteins that were stereoselectively engaged by 3-amino-azetidine acrylamides (shown for 20/50 μM and 3 h conditions). Data are from a single experiment representative of two independent experiments.

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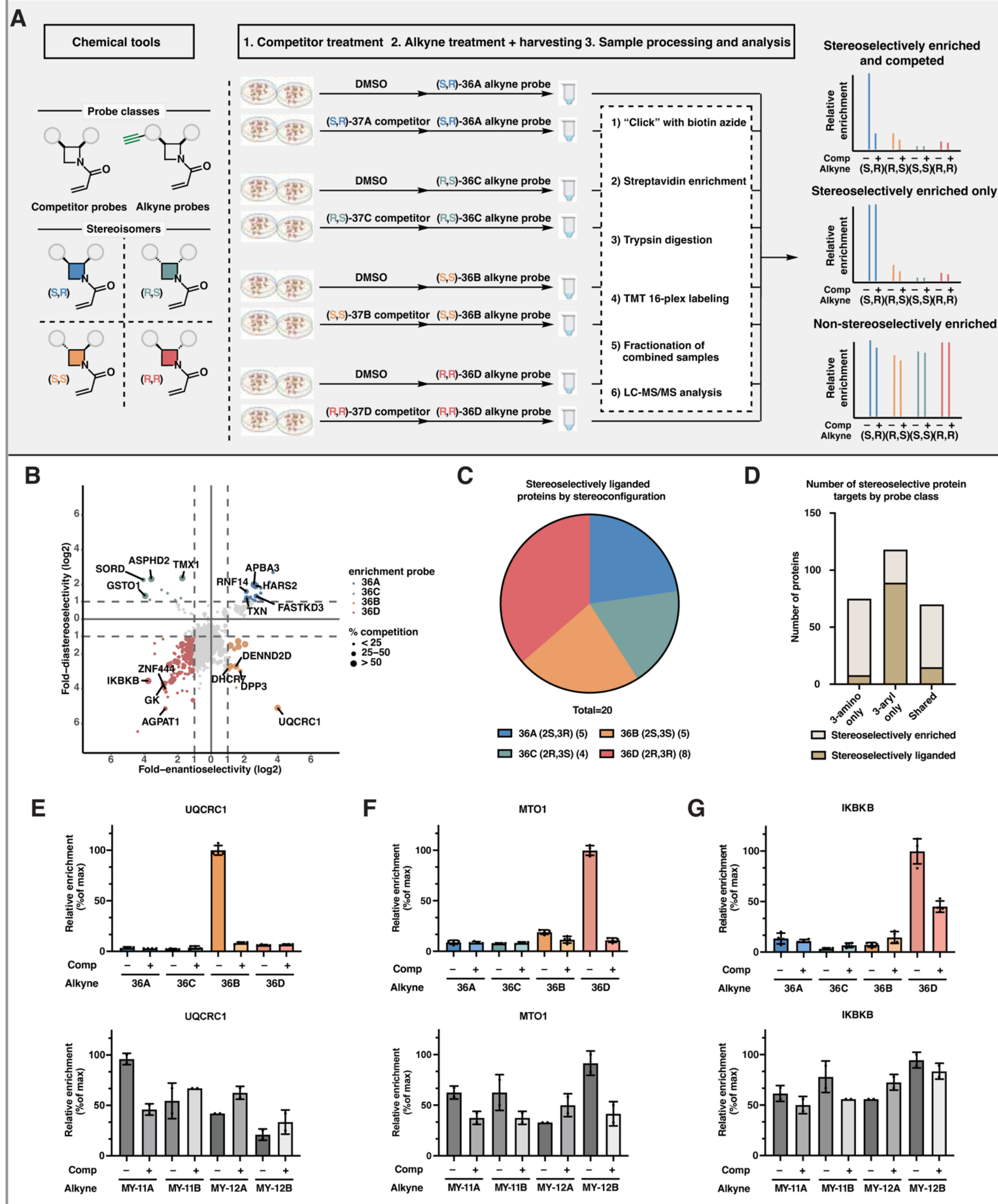

Figure 4. Protein-directed MS-ABPP of 3-amino stereoprobes in Ramos cells (20 µM competitor probes **37A–D** or DMSO for 2 h, 10 µM alkyne probes **36A–D** for 1h). (A) Workflow for protein-directed ABPP experiments where the stereoselective enrichment of proteins by alkyne stereoprobes and blockade of this enrichment by corresponding non-alkyne competitor stereoprobes are determined by multiplexed (TMT16plex) MS-based proteomics. (B) Quadrant plots highlighting enantio- and diastereoselectively enriched proteins for each stereoconfiguration of alkyne stereoprobes in Ramos cells. For each protein, enrichment enantioselectivity and diastereoselectivity (log2) are shown for the probe leading to the highest enrichment.

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Enantioselectivity is defined as the ratio of enrichment for one stereoisomer versus its enantiomer, and diastereoselectivity as the ratio of enrichment of one stereoisomer versus the average of its two diastereomers. For the quadrant plots, a protein is shown in color if both the enantioselectivity and diastereoselectivity of enrichment (log2) are greater than 1. (C) Pie chart showing the total number of proteins stereoselectively liganded by each stereoisomer of 3-amino-azetidine acrylamide stereoprobes. A protein is defined as being stereoselectively enriched if the enantioselectivity is greater than 3-fold; a protein was designated as being "liganded" if stereoselectively enriched and if pretreatment by the corresponding competitor probe resulted in >50% blockade of this enantioselective enrichment as compared to pretreatment with DMSO. (D) Bar graph comparing the total number of proteins stereoselectively enriched (lighter tan) and liganded (darker tan) by 3-amino stereoprobes vs 3-aryl stereoprobes in Ramos cells (treated with 5 μM of alkynylated 3-aryl stereoprobes and 10 μM of alkynylated 3-amino stereoprobes; pretreatment with 20 μM of either 3-aryl- or 3-amino competitor stereoprobes). A stereoselectively enriched protein was considered "shared" by the 3-amino and 3-aryl stereoprobes if it was enriched with >3-fold enantioselectivity by one stereoprobe chemotype and >2-fold enantioselectivity by the other stereoprobe chemotype. A liganded protein was considered "shared" if it was stereoselectively enriched and competed by >50% by one stereoprobe chemotype and >30% by the other stereoprobe chemotype. All other stereoselectively enriched or liganded targets of the 3-amino and 3-aryl stereoprobes were considered chemotype-selective. (E, F, G) Bar graphs showing protein-directed ABPP data for UQCRC1, MTO1, and IKBKB each of which was stereoselectively liganded by 3-amino stereoprobes, but not 3-aryl stereoprobes.

In conclusion, a modular approach enabling rapid access to enantiopure 2,3-disubstituted azetidines with either cis- or trans-orientation has been disclosed. The protocols outlined herein allow for installation of a diverse array of functionalization patterns as well as the creation of products with fully substituted stereocenters. A wide array of azetidines was prepared in addition to a complete set of acrylamide stereoprobes for use in chemical proteomic studies that revealed distinct protein interactions for 3-aryl- vs 3-amino stereoprobes in human cancer cells. Future studies can include expanding the complexity of accessible azetidines even further (higher order substition), mapping the liganded cysteines on 3-amino stereoprobe targets by cysteine-directed ABPP,[12] and understanding the functional impact of these interactions.

ASSOCIATED CONTENT

The Supporting Information is available free of charge.
Detailed experimental procedures and analytical data (PDF)
X-ray crystallographic data for **2b'**
X-ray crystallographic data for **2d**
X-ray crystallographic data for **13-**Trans
X-ray crystallographic data for **18-**Cis
X-ray crystallographic data for **18-**Trans
X-ray crystallographic data for **21-**Trans
X-ray crystallographic data for **28'**

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