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Article

A Structurally Diverse Compound Screening Library to Identify Substrates for Diamine, Polyamine, and Related Acetyltransferases

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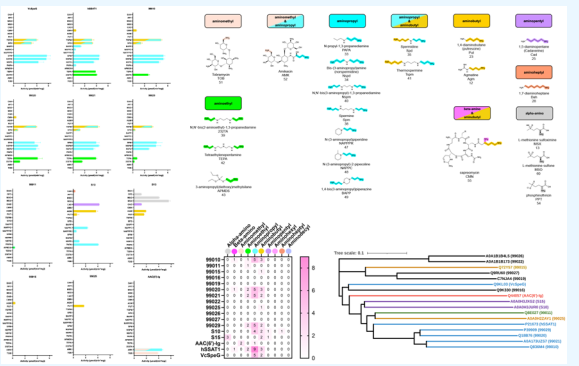
ABSTRACT: Spermidine/spermine *N*-acetyltransferases (SSATs) and other types of polyamine acetyltransferases (PAATs) acetylate diamines and/or polyamines. These enzymes are evolutionarily related and belong to the Gcn5-related *N*-acetyltransferase (GNAT) superfamily, yet we lack a fundamental understanding of their substrate specificity and/or promiscuity toward different compounds. Many of these enzymes are known or are predicted to acetylate polyamines, but in the cell there are other types of compounds that contain moieties derived from polyamines that may be the native substrates for these enzymes. To learn more about the identity of substrates that are acetylated, we selected and screened 17 different GNAT enzymes for activity toward a set of structurally diverse compounds that contained different types of amine moieties (*e.g.*, aminopropyl, aminobutyl, *etc.*). These compounds included diamines, triamines, and polyamines containing primary amino groups, and they had structural diversity with variation of the chain length and presence or absence of internal amino groups and other functional groups. We found 12 of the 17 enzymes acetylated at least one of the compounds. Some enzymes were selective toward acetylating only one compound while others exhibited substrate promiscuity toward numerous compounds. Our experimental results ultimately allowed us to pinpoint specific substrates that could be further investigated to more fully understand substrate specificity versus promiscuity of GNAT enzymes and the role of acetylated small molecules in cells.



INTRODUCTION

Gcn5-related *N*-acetyltransferases (GNATs) are exquisite model enzymes used to study protein evolution because they share a common structural fold; however, they generally exhibit low sequence similarity. These enzymes transfer an acyl group from a donor molecule, such as acetyl coenzyme A (AcCoA), to a terminal primary amino group in an acceptor molecule. The donor and acceptor binding sites are located in two distinct pockets on either side of a β-bulge within a centralized β-sheet. Acceptor substrates are diverse and range from numerous smaller organic molecules to larger macromolecules.[1−3] They play important roles in antibiotic resistance, post-translational modifications, and many other cellular processes,[1,2,4] but many of their functions remain unknown or underexplored. In the past, we sought to expand our knowledge of GNAT functions by developing a small molecule acceptor substrate screening assay.[5] We used the screening assay as a starting point to identify general classes of compounds that were substrates for previously uncharacterized proteins or proteins of unknown function. This assay uses a discontinuous two-step process. First, the GNAT enzyme is allowed to react with AcCoA and a panel of potential acceptor substrates and the reaction is then terminated by unfolding the protein with guanidine HCl. Second, the CoA produced from the enzymatic acylation assay reacts in a 1:1 ratio with Ellman's reagent (DTNB; 5,5′-dithiobis(2-nitrobenzoic acid)); the absorbance of the product of this reaction (TNB^{2-}) is then measured at A_{415nm}. We have used this screen to expand our knowledge about the structure/function relationships of many GNAT proteins,[6−13] but the initial panel of compounds was not fully representative of all GNAT functional capabilities we have explored.

One example of GNAT structural/functional studies that were launched in our laboratory using the results of the previous broad screening assay included the SpeG spermidine/spermine *N*-acetyltransferases (SSATs). These enzymes acetylate polyamines and there is interest in learning more about their roles in stress responses, bacterial biofilms, the human gut microbiome, and antibiotic resistance. We have

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Table 1. Compounds in the Screening Assay[a]

#	Chemical name (alternate name)	Abbreviation	Substituent
52	Amikacin	AMK	aminomethyl and aminopropyl
51	Tobramycin	TOB	aminomethyl
1	3-Aminopropionoic acid (β-alanine)	B-Ala	aminoethyl
43	3-Aminopropyl(diethoxy)methylsilane	APMDS	aminoethyl
39	*N,N′*-Bis(2-aminoethyl)-1,3-propanediamine	232TA	aminoethyl
42	Tetraethylenepentamine	TEPA	aminoethyl
45	Tyramine	Tyram	aminoethyl
49	1,4-Bis(3-aminopropyl)piperazine	BAPP	aminopropyl
41	1,4-Butanediamine (Thermospermine)	Tspm	aminopropyl and aminobutyl
46	3-Phenyl-1-propylamine (3-phenylpropylamine)	3P1PA	aminopropyl
2	4-Aminobutyric acid (γ-Aminobutyric acid)	GABA	aminopropyl
34	Bis(3-aminopropyl)amine (Norspermidine)	Nspd	aminopropyl
3	Ethyl 4-aminobutyrate	E4AB	aminopropyl
8	L-2,4-diaminobutyric acid	DAB	aminopropyl and alpha-amino
48	*N*-(3-Aminopropyl)-2-pipecoline	NAPPC	aminopropyl
47	*N*-(3-Aminopropyl)piperidine	NAPPPR	aminopropyl
30	*N*-Boc-1,3-propanediamine	Boc13PDA	aminopropyl
33	*N*-Propyl-1,3-propanediamine	PAPA	aminopropyl
40	*N,N′*-Bis(3-aminopropyl)-1,3-propanediamine (Norspermine)	Nspm	aminopropyl
61	Polymyxin B	PolyB	aminopropyl
35	Spermidine	Spd	aminopropyl and aminobutyl
38	Spermine	Spm	aminopropyl
23	1,4-Diaminobutane (Putrescine)	Put	aminobutyl
19	3,6-diaminohexanoic acid (β-lysine)	BLys	aminobutyl and beta-amino
4	5-Aminovaleric acid	5AVA	aminobutyl
12	Agmatine	Agm	aminobutyl
59	Atosiban	Atosiban	aminobutyl
53	Bacitracin	Bacit	aminobutyl
55	Capreomycin	CMN	aminobutyl and beta-amino
37	(2S)-Hypusine	Hyp	aminobutyl and alpha-amino
9	L-ornithine	Orn	aminobutyl and alpha-amino
24	*N*-Acetylputrescine	AcPut	aminobutyl
10	*N*-Alpha-Acetyl-L-ornithine	AcOrn	aminobutyl
31	*N*-Boc-1,4-butanediamine	Boc14BDA	aminobutyl
32	*N*-Fmoc-1,4-butanediamine	Fmoc14BDA	aminobutyl
36	N^1,N^4-Bis-Boc-spermidine	NNBisBocSpd	aminobutyl
25	1,5-Diaminopentane (Cadaverine)	Cad	aminopentyl
5	6-Aminohexanoic acid (6-aminocaproic acid)	6AHexA	aminopentyl
57	Deferoxamine	Deferox	aminopentyl
18	DL-5-Hydroxylysine	5HLys	aminopentyl and alpha-amino
44	D-Lysine	DLys	aminopentyl and alpha-amino
20	L-beta-Homolysine	BHomoLys	aminopentyl and beta-amino
15	L-lysine	Lys	aminopentyl and alpha-amino
16	*N*-Alpha-acetyl L-lysine	NaAcLys	aminopentyl
21	*N*-Alpha-Boc-L-lysine (Boc-Lys-OH)	NaBocLys	aminopentyl
22	*N*-Alpha-Cbz-L-lysine thiobenzyl ester	NaCbzLys	aminopentyl
26	*N*-Boc-cadaverine	BocCad	aminopentyl
27	*N*-Fmoc-cadaverine	FmocCad	aminopentyl
14	S-(2-Aminoethyl)-L-cysteine (Thialysine)	Thia	aminopentyl and alpha-amino
58	Thymopentin	Thymo	aminopentyl
6	7-Aminoheptanoic acid	7AHepA	aminohexyl
7	*N*-Fmoc-1,6-hexanediamine	F16HexDA	aminohexyl
28	1,7-Diaminoheptane	Dah	aminoheptyl
29	1,10-Diaminodecane	110DAD	aminodecyl
11	Boc-Orn-OH (Boc-L-ornithine)	BocOrn	alpha-amino
54	DL-Phosphinothricin	PPT	alpha-amino
60	L-Methionine sulfone	MSO	alpha-amino
13	L-Methionine sulfoximine	MSX	alpha-amino
50	*N*-Epsilon-acetyl-D-lysine	NeAcDLys	alpha-amino
17	*N*-Epsilon-acetyl L-lysine	NeAcLys	alpha-amino
56	Streptothricin (Nourseothricin)	Strepto	beta-amino

[a]The compounds listed are organized by type of substituent and the numbers correspond to compounds listed in Table S2. Substituents are colored based on chain length and are consistent across figures in the manuscript. Those compounds with two different types of amine moieties have a colored outline in addition to fill. The table is sorted by type of substituent rather than #.

studied the SpeG enzyme from a variety of Gram-negative (*Escherichia coli* (EcSpeG) and *Vibrio cholerae* (VcSpeG)) and Gram-positive (*Staphylococcus aureus* (SaSpeG) and *Bacillus thuringiensis* (BtSpeG)) bacteria and found that they acetylate spermidine (Spd) and spermine (Spm) with a general preference for Spm over Spd.[11,14−16] Interestingly, the enzyme's ability to acetylate these polyamines is not necessarily consistent with the identity of polyamines that are natively produced within these bacteria. For example, the VcSpeG enzyme acetylates Spd and Spm, which must be imported if the

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enzyme were to encounter and acetylate these compounds;[17,18] however, norspermidine (Nspd) is the predominant polyamine in *V. cholerae*[19]. EcSpeG also acetylates Spm and Spd,[16,20] but Spd and putrescine (Put) are the predominant polyamines in *E. coli*. On the other hand, *S. aureus* is a polyamine auxotroph, and the SaSpeG enzyme acetylates Spm and Spd,[14,21] which also must be imported for the enzyme to acetylate these compounds *in vivo*. It may be that the SpeG enzyme in different bacteria has retained a conserved set of residues that enable it to acetylate diverse polyamines encountered in various environments. To determine the substrate specificity and promiscuity of these and other SSAT enzymes more fully, it became necessary to more broadly examine different types of polyamines that could be environmentally relevant. This led us to explore the enzyme activity of a select set of GNAT enzymes toward an expanded panel of substrates, which included diverse polyamines and other compounds that have substituents that resemble polyamines.

A key motivator in our decision to screen a diverse library of compounds was the ability to determine whether these enzymes could use other types of polyamines that are present in different organisms or in different types of environments. This is important because our studies have shown that some GNAT enzymes, including SSATs, exhibit substrate specificity for compounds that are not inherently present within the organism in which the GNAT is encoded. Another motivator was that we were curious whether GNATs have an inherent ability to acetylate numerous polyamines (substrate promiscuity) and/or if they maintain a rigid substrate specificity. This was based on the knowledge that some GNAT proteins have exhibited substrate promiscuity toward lysine residues on proteins, polyamines, and/or aminoglycoside antibiotics.[22−26] Thus, there may be an underlying ability or historical code that is inherent in some GNAT protein active sites which enables this type of promiscuity or specificity to occur. Finally, we were motivated to determine whether utilizing an expanded compound library would provide insight into the possibility of GNATs acetylating other compounds like siderophores and antibiotics, which have substituents that resemble polyamines or structural moieties of polyamines. Ultimately, our decision to utilize a more diverse library of compounds rather than systematic and targeted examination of GNAT substrate structural changes of polyamines alone (*e.g.*, focusing on varying chain length or di-, tri-, tetra-amines) was driven primarily by the opportunity to identify common structural features on unique chemical scaffolds that enable specificity in GNAT-mediated substrate acetylation. However, our library does incorporate some polyamine compounds that can be used to address systematic changes in length or quantity of internal amines. In this study, we tested several characterized and uncharacterized GNAT enzymes from our laboratory to determine their activity toward these compounds. Our results expanded our knowledge of the structural elements in polyamines that are recognized by GNATs and we learned more about which complex substrates are acetylated by these enzymes. Exploring the functional landscape of a wider variety of GNATs will ultimately lead to a more thorough understanding of the evolution of substrate specificity and promiscuity for polyamines and beyond in this intriguing family of proteins.

RESULTS

Selection of Compounds as Potential Acceptor Substrates. The compounds that were selected for this screen were based on their chemical structures, hazardous properties, solubility, purity, and costs. While we sought to examine GNAT enzyme activity toward a systematically varied series of compounds based on chain length and presence of internal amines, not all compounds for this ideal series were readily available and limited our ability to query all types of structural changes in various scaffolds. Below is the rationale for compound selection and exclusion in the assay. The full list of 143 compounds that were initially considered for this assay are shown in Table S1, and the final 61 compounds that were selected and screened in this study are shown in Table 1 and Figure S1 and Table S2. Substituents were classified based on the number of contiguous carbons or heteroatoms between the primary amino group and either a branching point or a nitrogen atom. If multiple amino groups were present, the most sterically accessible aminoalkyl substituent was selected.

Structures. We searched commercially available chemical supplier catalogs (Millipore Sigma and Santa Cruz Biotechnology) for compounds that had accessible primary amino groups (*e.g.*, aminoethyl, aminopropyl, substituents) and had been identified as substrates or could theoretically be acetylated by GNAT enzymes. Compounds that had internal amines, *e.g.*, triamine, tetramine, etc. or antibiotics with terminal accessible primary amine substituents were selected; compounds without any primary amine substituents were excluded. We hypothesized that incorporating compounds with different hydrophobicity and charge might expand our ability to identify GNAT substrates. Our rationale for including some compounds, such as those typically used for organic synthesis reactions (*e.g.*, tert butyl, Fmoc), was to test compounds that had high hydrophobic character on one end of the molecule while still maintaining a positive charge on the other end of the molecule. This was based on the hypothesis that some GNATs may recognize aminopropyl or other types of substituents on compounds that have similar characteristics but are more complex, such as siderophores, antibiotics, or proteins. Furthermore, these substrates could provide a starting point for designing inhibitors or other types of substrates for these types of proteins.

Hazards, Solubility, Purity, Cost, and Other Considerations. We collected information about the hazardous properties of the selected compounds from the safety data sheets and removed those that were considered fatal upon contact or were deemed to be particularly dangerous *via* skin contact, eye damage, and/or inhalation or required atypical safety measures. However, we did include some hazardous compounds, such as Nspd and Put, because of the value they added to the overall screen and the fact they are known substrates for some GNAT enzymes. All screening assays were performed in the fume hood out of an abundance of caution. We also excluded compounds that were only soluble in high concentrations of organic solvents or were incompatible with our assay (*e.g.*, DMSO, which reacts with DTNB and leads to a high background absorbance). The partition coefficient (log *P*) values from PubChem were used to identify compounds with values between 9.6 to 3.6. We preferred compounds that were soluble in water, but we did include some compounds that were soluble in 50% ethanol so that the final concentration of ethanol in the final reaction was no higher than 1−2%. Our

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prior studies have shown this concentration of ethanol is tolerated by many GNAT enzymes. Only compounds that had ≥95% purity were selected. Additionally, nonvalidated compounds, such as those listed as Aldrich^{CPR} in Sigma's catalog, and particularly cost prohibitive compounds were also removed from consideration. During our initial experiments we included two compounds, daptomycin and caspofungin but they precipitated during the enzymatic reaction step of the assay and were considered unreliable indicators of activity. It is possible these compounds were indeed substrates for some enzymes, but acetylation would reduce the charge of the molecules, which would then lessen their solubility. Due to this uncertainty, we excluded these compounds from the final screen.

Selection of Proteins for Screening. We selected 17 GNAT proteins across different bacteria from emerging or re-emerging pathogens, stress resistant organisms, or other human health-relevant species to expand our understanding of what types of polyamines and molecules with similar moieties could be acetylated (Table 2). To test the versatility of the assay, we selected several uncharacterized or underexplored GNATs that either had between 20−28% sequence similarity to the VcSpeG SSAT protein or were in our collection of clones that had not yet been characterized. We included VcSpeG[11] and hSSAT1,[27] which are SSATs and acetylate polyamines, as well as the AAC(6′)-Ig protein[28] that acetylates aminoglycosides as positive controls. Throughout the manuscript, we refer to the proteins using internal IDs listed in Table 2 due to the length and complexity of UniProtID identifiers.

Table 2. Proteins Screened for Enzymatic Activity and Their Corresponding Identification Numbers and Cloning Vectors

internal protein ID	UniProtID	organism	vector
99010	Q836M4	*Enterococcus faecalis V583*	pMCSG53
99011	Q8E027	*Streptococcus agalactiae A909*	pMCSG53
99015	Q72Y57	*Bacillus cereus ATCC 10987*	pMCSG53
99016	Q9K330	*Vibrio cholerae O1 biovar El Tor str. N16961*	pMCSG53
99019	C7NJA4	*Kytococcus sedentarius DSM 20547*	pMCSG53
99020	Q18B76	*Clostridioides difficile 630*	pMCSG53
99021	A0A173UZS7	*Blautia hydrogenotrophica*	pMCSG53
99022	A0A1B1B173	*Streptomyces griseochromogenes*	pMCSG53
99025	A0A0H2ZAY1	*Pseudomonas aeruginosa* UCBPP-PA14	pMCSG53
99026	A0A1B1B4L5	*Streptomyces griseochromogenes*	pMCSG53
99027	Q9RU60	*Deinococcus radiodurans R1*	pMCSG53
99029	P39909	*Bacillus subtilis subsp.. subtilis str. 168*	pMCSG53
S10	A0A0H3JUR0	*Staphylococcus aureus (strain Mu50)*	pMCSG21
S15	A0A0H3JXG2	*Staphylococcus aureus (strain Mu50)*	pMCSG21
AAC(6′)-Ig	Q44057	*Acinetobacter hemolyticus*	p15Tv-LIC
hSSAT1	P21673	*Homo sapiens*	pMCSG53
VcSpeG	Q9KL03	*Vibrio cholerae*	pMCSG7

Protein Activity. We screened a total of 17 proteins for activity toward 61 different compounds and found 12 of these proteins acetylated at least one compound in the screen. Of the 61 compounds tested, 22 of them were identified as substrates. These compounds represent a variety of amino substituents including α-amino, β-amino, aminomethyl, -ethyl, -propyl, -butyl, -pentyl, and -heptyl groups (Figure 1). While compounds with aminopentyl substituents were dominant in the set of 61 compounds that were tested (Figure 2), we found that compounds with aminopropyl substituents were more frequently identified as substrates (Figures 1 and 2). Next, we grouped the 12 active enzymes into two main groups of 6 proteins based on their substrates to determine whether patterns in substrate usage emerged (Figures 3 and 4). The remaining 5 proteins that did not exhibit activity toward any compounds in the screen included 99016, 99019, 99022, 99026, and 99027 and were excluded from further analysis.

The first main group of 6 proteins, including 99010, 99020, 99021, 99029, and positive controls hSSAT1 and VcSpeG proteins, exhibited similar patterns of substrate usage (Figure 3). The compounds that were identified as substrates for these enzymes included those with aminoethyl, aminopropyl, and aminobutyl substituents or amino substituents α or β to a carbonyl group. Substrates with aminoethyl substituents included tetraethylenepentamine (TEPA) and *N*,*N*′-bis(2-aminoethyl)-1,3-propanediamine (232TA), which are tetra- and pentaamines. In contrast to hSSAT1, the VcSpeG enzyme did not acetylate any of the aminoethyl substituent compounds. The extent to which these compounds were acetylated by 99010, 99020, 99021, and 99029 proteins varied. For example, 99010 did not acetylate TEPA and the activity of 99029 toward TEPA and 232TA was significantly reduced.

Two major sets of compounds with only aminopropyl or both aminopropyl and aminobutyl substituents were also identified as substrates for the first group of proteins (Figure 3). All of these compounds were tri- and tetraamines. The compounds that contained only aminopropyl substituents included Spm, Nspd, and norspermine (Nspm) and were consistently acetylated by VcSpeG, hSSAT1, 99010, 99020, 99021, and 99029 proteins (Figure 3). Additionally, hSSAT1 was the only protein from this group that acetylated compounds that had heterocyclic components within their structures: 1,4-bis(3-aminopropyl)piperazine (BAPP), *N*-(3-aminopropyl)-2-pipecoline (NAPPC), and *N*-(3-aminopropyl)piperidine (NAPPPR) substrates. hSSAT1 also acetylated the compound *N*-propyl-1,3-propanediamine (PAPA), which has a single free aminopropyl group in its structure. These compounds were not used as readily as other long-chain polyamines. Substrates with both aminopropyl and aminobutyl substituents were tri- and tetraamines and included Spd and thermospermine (Tspm). A consistent observation is that while Spd and Tspm were used similarly by VcSpeG and hSSAT1, the 99010, 99020, 99021, and 99029 enzymes showed less activity toward Spd compared to Tspm. Further liquid chromatography and/or mass spectrometry studies are warranted to determine which terminal amine on these compounds is being acetylated. We also observed lower activity toward capreomycin (CMN), which has β-amino and aminobutyl substituents, for several proteins (hSSAT1, 99010, and 99020). Overall, we found substrates with aminopropyl substituents appear to be more widely acetylated than other compounds in the screen for the VcSpeG, hSSAT1, 99010, 99020, 99021, and 99029 enzymes. There were no trends

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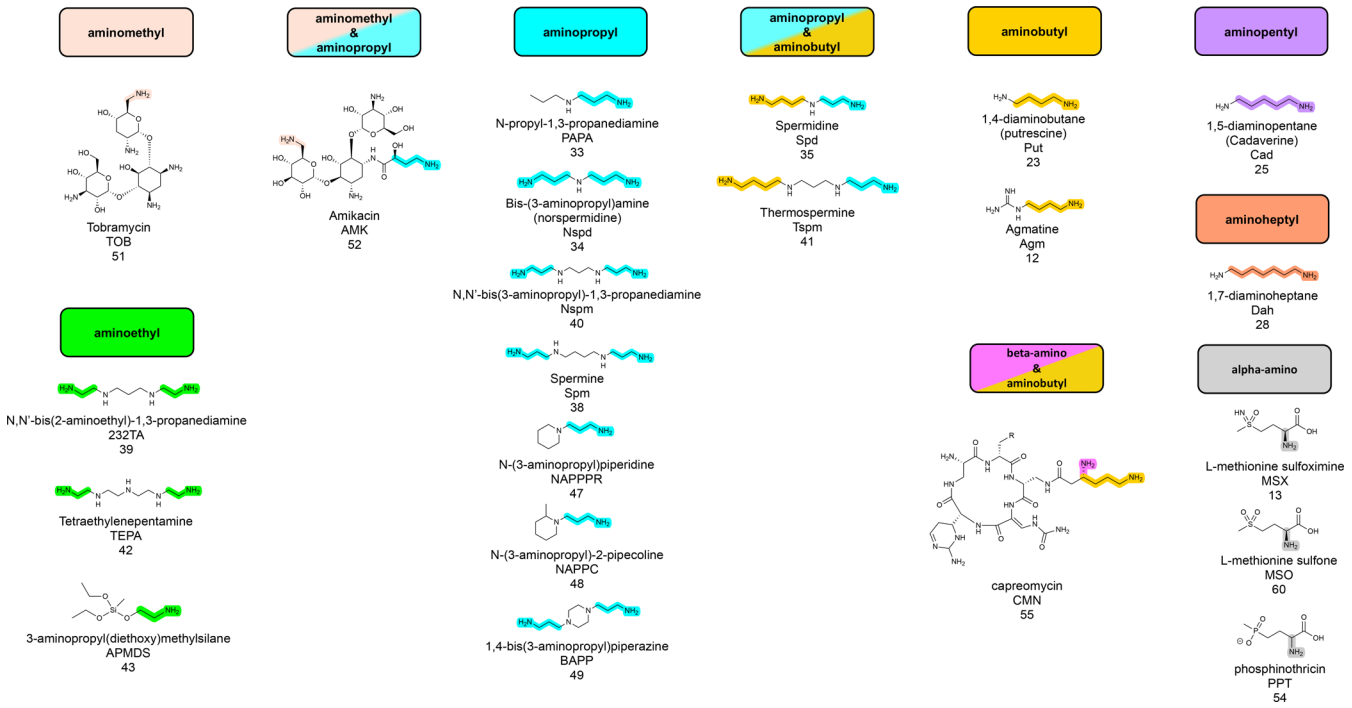

Figure 1. Breadth of compounds identified as substrates for GNATs screened. Structures of compounds identified as acetylated are grouped and colored by type of substituent. The structures of compounds that fit into multiple categories are indicated with color gradient boxes. Note, only compounds identified as acetylated are shown here. All other compounds in the screening assay and their structures are shown in Figure S1.

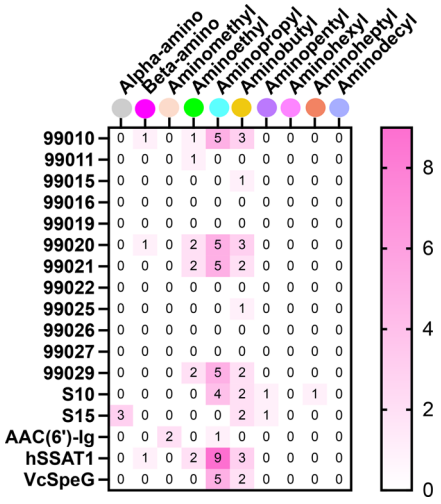

Figure 2. Frequency of types of substituents on substrates acetylated by enzyme. The heatmap shows the frequency of acetylation of various substrates by substituent type. Substituents are indicated above the columns and are colored as in other figures in the manuscript. If a compound has more than one type of substituent it is counted in both categories since we cannot confirm which site is acetylated. Protein identifiers are indicated by row. See Tables 1 and 2 for more details.

observed when we tried to classify the substrates by di-, tri-, tetra-amine, etc. rather than aminomethyl, -ethyl, -propyl, etc. Thus, it appears the enzymes are recognizing length of amino substituent rather than presence of a certain number of internal amines.

The second main group of proteins, 99011, S10, S15, 99015, 99025, and AAC(6′)-Ig, showed a different pattern for the types of substrates that were acetylated (Figure 4). For example, the AAC(6′)-Ig control was the only protein that acetylated compounds with aminomethyl substituents. These compounds included the aminoglycoside antibiotics tobramycin and amikacin. The only protein in this group that acetylated a compound with an aminoethyl substituent was 99011, albeit with low activity. This substrate, aminopropyl-(diethoxy)methylsilane (APMDS), has a single aminoethyl group and was not acetylated by any other enzyme that was tested. Unlike the first main group of proteins that showed a preference for long-chain polyamines with multiple aminopropyl groups, the S10 enzyme acetylated compounds that had predominantly single aminopropyl substituents. For example, it acetylated NAPPC, NAPPPR, Nspd, and PAPA. Additionally, substrates with α amino groups, including phosphinothricin (PPT), L-methionine sulfone (MSO), and L-methionine sulfoximine (MSX), were only acetylated by the S15 enzyme. Similar to the first group of proteins, we did not observe any trends in substrate acetylation when we classified substrates by internal amine: substrate utilization appears to be based on aminoalkyl substituent length.

Enzymes that acetylated compounds with aminobutyl substituents in the second main group of proteins included S10, S15, 99015, and 99025. Both Put and agmatine (Agm) were acetylated by S10 and S15 enzymes; however, S10 acetylated Agm more readily than Put compared to the S15 enzyme. In contrast, 99015 only acetylated Agm and 99025 only acetylated Put. Very little activity was observed for the 99015 enzyme. S10 and S15 proteins were the only ones that acetylated compounds with aminopentyl substituents: S10 and S15 both acetylated cadaverine (Cad). Low activity was observed for S15 toward the only substrate with an aminoheptyl substituent: 1,7-diaminoheptane (Dah). Overall, except for the S10 and S15 enzymes, it appears the enzymes in this second main group were more selective.

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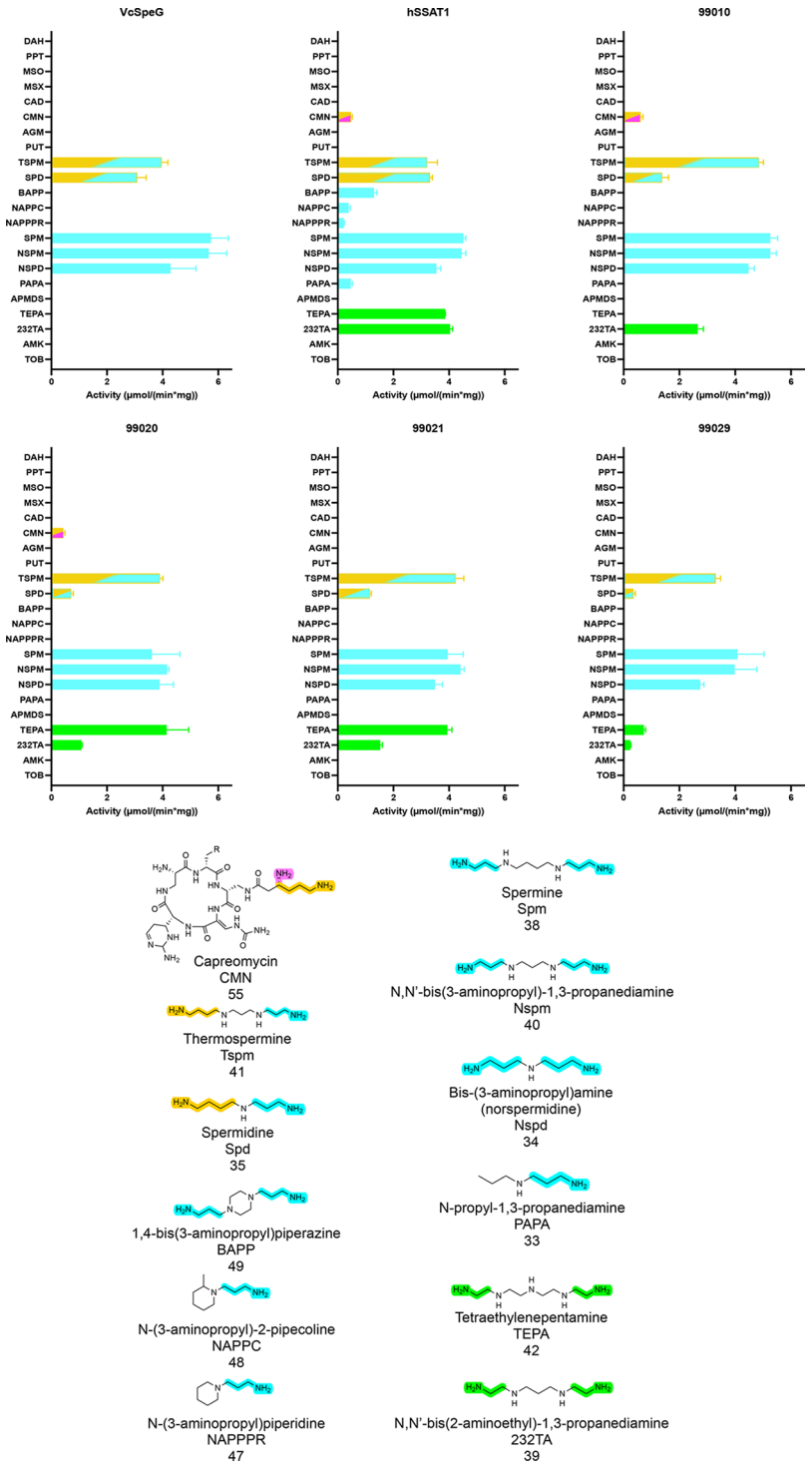

Figure 3. Enzymatic activity of first set of 6 proteins toward a panel of 61 compounds. Only 22 of the 61 compounds were identified as substrates across all proteins tested and are shown on the *y*-axis. All compounds and their corresponding abbreviations are indicated in Table 1. Error bars show the standard deviation of duplicate reactions. Bars on the graphs are colored based on different substituents: α-amino (gray), β-amino (magenta), aminomethyl (pink), aminoethyl (green), aminopropyl (cyan), aminobutyl (gold), aminopentyl (purple), and aminoheptyl (salmon). Compounds containing two different moieties are represented with two color gradients. Prism v10.2.3 was used to generate these plots, but Microsoft PowerPoint was used to make gradient overlays. Structures of compounds and corresponding names and abbreviations are also shown.

DISCUSSION

Versatility of the Screen. Our results provide new functional insight for GNAT enzymes that will be helpful for future computational predictions of protein functions. The untapped functional space for uncharacterized proteins is vast, and it is likely that additional substrates exist for evolutionarily related GNAT enzymes. The results of this study provide a first look at the types of molecules that can be modified by this subset of selected proteins, which is important as we try to identify functions and associate them with uncharacterized proteins from the GNAT family. Functional validation of more

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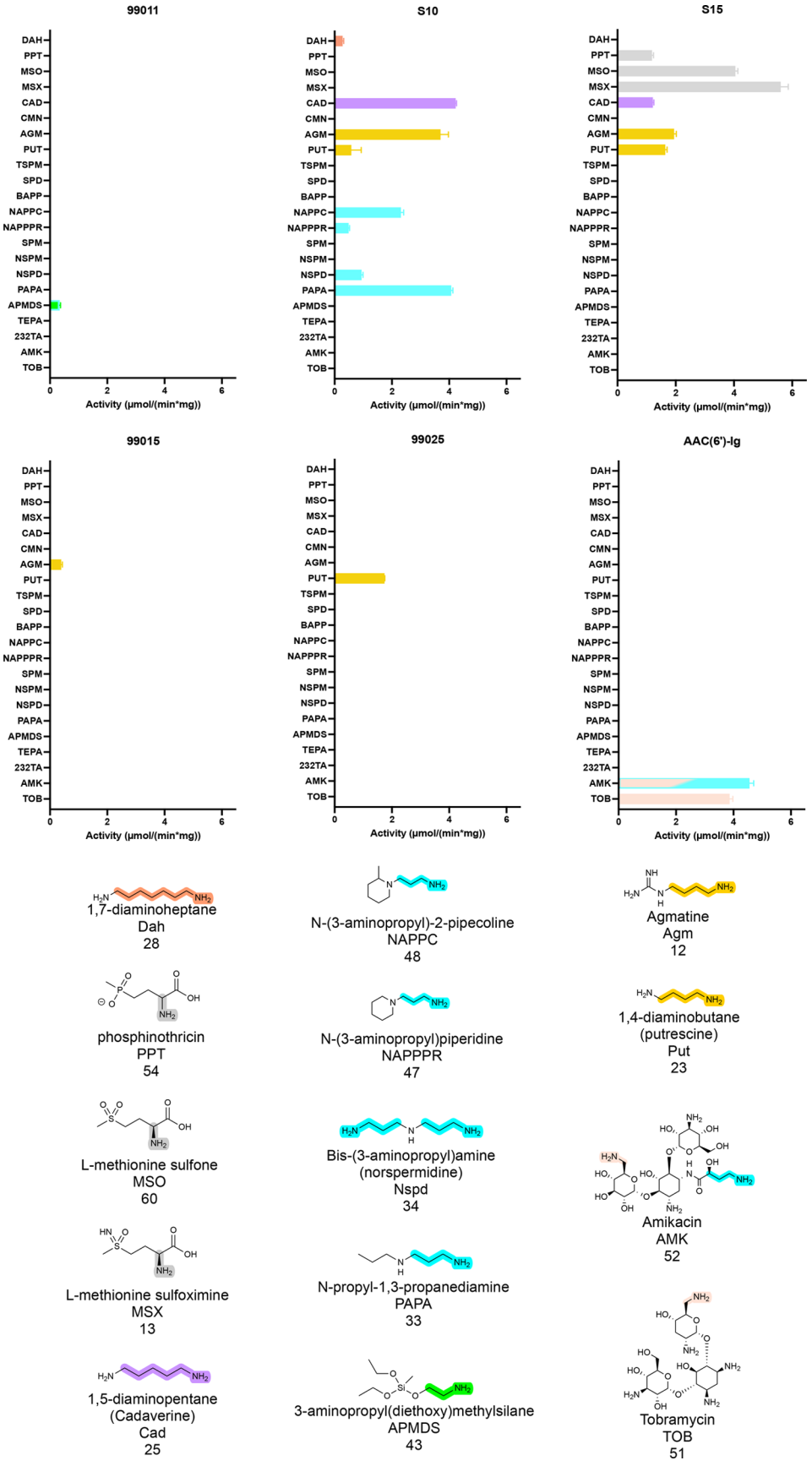

Figure 4. Enzymatic activity of second set of 6 proteins toward a panel of 61 compounds. Content and colors are consistent with those described in Figure 3.

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enzymes *in vitro* and *in vivo* is particularly relevant to improve the accuracy for annotations of protein functions computationally.

The panel of compounds we constructed in this study was versatile for identifying novel substrates for GNAT enzymes *in vitro*. We showed that over 1/3 of these compounds were substrates for 12 of the enzymes that we tested. Some enzymes selectively acetylated single compounds, whereas other enzymes were more promiscuous and active toward a variety of compounds (Figures 2−4). We found most of the enzymes that we tested preferred substrates with aminopropyl substituents (Figure 2). Compounds with aminoethyl and aminobutyl substituents were also utilized more readily than compounds with other lengths of amino substituents. Additionally, not all compounds with aminopropyl substituents were acetylated, which indicates there is some level of substrate specificity even for enzymes that appear promiscuous. This could be due to an inherent set of residues that are critical for substrate recognition and binding within the enzyme active sites. Indeed, a cursory phylogenetic analysis of these protein sequences paired with their substrate preferences shows that some proteins group together by substrate utilization, but others are disparate (Figure 5). Future structural and functional studies are required to tease out these details.

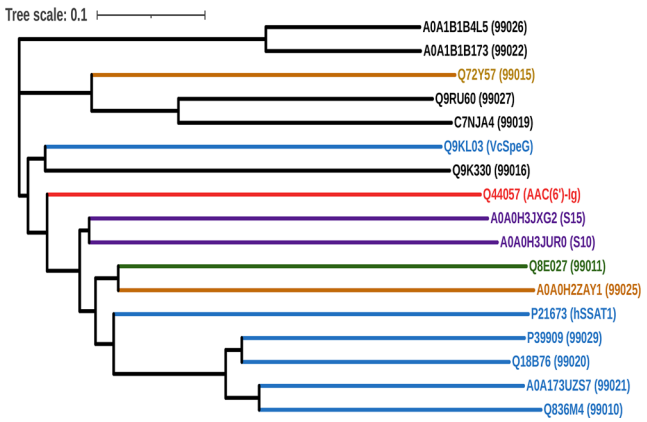

Figure 5. Phylogenetic tree. Protein sequences for all 17 GNAT proteins assayed in this study were obtained and aligned using the UniProt server (https://www.uniprot.org) and the resulting phylogenetic tree from the multiple sequence alignment was downloaded for further analysis. The unrooted tree was then visualized using iTOL (https://itol.embl.de/). Branches are labeled with UniProt IDs and internal protein IDs (in parentheses) as described in Table 2. Colored branches correspond to groups of proteins with similar activity profiles: Proteins in Figure 3 with aminopropyl and other types of substituents (VcSpeG, hSSAT1, 99010, 99020, 99021, and 99029; blue), proteins in Figure 4 with substrates with aminopentyl and other substituents (S10, S15; purple), aminobutyl substituents (99015, 99025; gold) aminoethyl substituents (99011; green), and aminomethyl substituents (AAC(6′)-Ig; red). Branches colored in black correspond to proteins without any identifiable substrate. Refer to Figures 3 and 4 for more specific information about substrate specificity. Scale represents substitutions per site.

While some of the enzymes we tested shared sequence similarity to SSATs and were predicted to acetylate polyamines, several did not acetylate any compounds in the screen. It is possible that some small molecule substrates mimic portions of larger proteins or other complex substrates, but they are unable to be turned over efficiently. A larger substrate would have additional interactions with the acetyltransferase that are required for recognition and efficient turnover. Alternatively, a lack of activity could indicate that a different function may exist for these proteins that is not represented in the compounds we tested. Regardless, our results are limited by the compounds we included in the screen in part due to their commercial availability, solubility, chemical interference with the assay, and toxicity. Further studies are required to elucidate whether enzyme inactivity is due to active site residue alterations compared to other SSATs and whether a different function exists. Here, we provide new information upon which additional studies can be developed to explore how GNAT enzymes have evolved to acetylate different types of compounds.

Observations and Caveats. As we designed the approach to this study, we incorporated lessons we had learned from our previous broad substrate screening assay and subsequent enzyme characterizations. For example, we reduced the concentrations of potential acceptor substrates from 2.5 to 1 mM because we found that in some cases the higher concentration was excessive and gave false positive results. While these types of screening assays can be used to identify substrates for further characterization, it is also important to recognize the limitations of this assay. For example, the concentrations of substrates screened during this assay and later steady-state kinetic characterization should be reasonable for the system. Identifying a "hit" in the screening assay does not necessarily mean the substrate will be preferred once steady-state kinetics are performed and different substrate specificities are compared. It could be that some enzymes have a low apparent affinity for different "hits" that is only revealed once steady-state kinetics are performed and enzyme concentrations are reduced. Furthermore, if the apparent affinity for an acceptor substrate is in the very high millimolar range, it is possible the substrate is not native. In this case, the screen was useful to provide clues for the types of substrates that are utilized by the enzyme, but further testing across a range of structurally similar compounds is required.

We also recognize that this screening assay is just the first step toward full characterization of these enzymes. Information that this assay does not provide includes: (1) the position of acetylation on the substrate (*i.e.*, it only measures production of CoA), (2) the apparent affinity of the substrates and substrate specificity (*i.e.*, substrate-saturation curves are required to deduce this information), and (3) the identity of the compound (*i.e.*, if a contaminant is present in the commercially supplied chemical, additional techniques would be required to confirm the identity of the substrate). However, the benefits of this assay include preliminary screening data that can be used as a foundation for further kinetic assays or to generate hypotheses about potential GNAT function. It also shows that while some GNAT enzymes share sequence identity to polyamine acetyltransferases, they do not always acetylate these types of molecules. Ultimately, substrate specificity for these enzymes is quite complex and warrants additional study.

Substrate Specificity and Promiscuity of GNATs and Implications for the Future. It is notoriously difficult to predict functions from sequences for GNAT enzymes. This is largely due to their low sequence identities for proteins with similar functions, a lack of experimental evidence of protein function across a diverse range of proteins, or their ability to acetylate multiple types of substrates (promiscuity). One

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example that is relevant to GNATs that acetylate polyamines includes the aaNAT5b GNAT protein from the *Aedes aegypti* mosquito. It was previously annotated as an arylalkylamine acetyltransferase based on its crystal structure and activity toward histamine.[29] Later, kinetic assays showed this enzyme acetylated the polyamine spermine significantly better than histamine, indicating its function may be more related to SSAT enzymes than aaNATs, or at least exhibit broader substrate promiscuity for both arylalkylamines and polyamines.[30] This concept has also been observed for other GNAT proteins that acetylate both histones and polyamines.[24,31]

Historically, SSAT enzymes have been shown to acetylate multiple types of polyamines and other substrates during functional characterization experiments, especially in the older literature. Yet, this concept remains largely deemphasized and underexplored even though hints of substrate promiscuity is widespread in this family of proteins. Here, we describe the two most prominent examples in the literature that also correspond to our positive controls in the assay: hSSAT1 and SpeG.

hSSAT1. The hSSAT1 protein acetylates a variety of different substrates *in vitro*, including diamines, polyamines, drugs, and proteins. In fact, Spm and Spd are the most well-described substrates and are selectively acetylated at the N^1 position; the N^8 position of Spd is not acetylated.[32] While these molecules are good substrates, the hSSAT1 protein actually acetylates Nspd and Nspm more readily.[33] hSSAT1 also acetylates shorter molecules such as diethylene triamine, ethylene diamine, and 1,3-diaminopropane; however, they are not good substrates compared to other longer chain polyamines.[34] Furthermore, chemo- and radiotherapy drugs deoxyspergualin and WR1065, which have aminopropyl substituents in their structures, are also acetylated by hSSAT1.[27] The copper chelator triethylenetetramine (TETA) is acetylated by the mouse SSAT1 protein, which is an hSSAT1 homologue.[35,36] Studies have also shown the hSSAT1 enzyme selectively acetylates a hypusinated lysine residue of the eIF5A transcription factor, but Spm and Spd inhibit hypusination and are better substrates than the hypusinated eIF5A protein.[37] The hypusine modification mimics Spd, which explains some of the observed substrate promiscuity. Thus, the hSSAT1 enzyme is versatile in its ability to acetylate numerous classes of substrates.

SpeG. Less is known about the substrate promiscuity of bacterial SpeG enzymes compared to hSSAT1. For example, the SpeG enzyme from a variety of different bacteria also acetylates Spm and Spd.[11,14,15,20,21] Unlike the hSSAT1 enzyme, SpeG generally acetylates both the N^1 and N^8 positions of Spd.[14,20] One exception is the VcSpeG enzyme, which exhibits a stronger preference for N^1 than N^8 on Spd.[14] The VcSpeG enzyme was also used in our prior broad substrate screening assay; the only substrates that were identified at that time were Spm, Spd and N^1-acetylspermine.[5] In this study, we have shown that the VcSpeG enzyme acetylates a broader spectrum of compounds than was previously recognized, including some overlapping substrate utilization compared to the hSSAT1 enzyme. Similarly, we have identified new compounds that are acetylated by hSSAT1. This promiscuity can be observed for many of the enzymes we tested that had similar substrate profiles to VcSpeG and hSSAT1 and shows the flexibility of the substrate recognition in this family of proteins.

Overall, our results have shown that the potential substrate specificity and promiscuity of the GNAT enzymes we tested vary widely. While we know these substrates are likely native for some enzymes, many are not, and instead show the diverse capability of these enzymes for recognizing different compounds. This is important in the context of how protein function is classically defined and what types of molecules the enzyme may encounter in different environments. For example, it is known that many proteins turn over distinct molecules to form specific products that are part of metabolic pathways or other systems within an organism (native function). However, many enzymes encounter different molecules that are not "native" and could be substrates due to their similar chemical properties. Non-native molecules may also be imported, encountered in a specific environment, or produced as byproducts of other cellular reactions. For instance, the native function of HDAC10 is to deacetylate lysine residues on histone proteins; however, this enzyme can also deacetylate N^8-acetylspermidine to form Spd when Spd biosynthesis is limited in specific environments.[38] Indeed, some enzymes with clearly defined functions, such as glycolytic enzymes, are also being recognized as playing roles in metabolic repair.[39] Perhaps the potential substrate promiscuity observed for some GNAT enzymes introduces additional flexibility for tuning their activity toward a variety of substrates that may be encountered in diverse environments or metabolic conditions.

CONCLUSIONS

This study provides a foundation upon which to further study the structure/function relationships and substrate specificity and promiscuity of diamine and polyamine acetyltransferases. Here, we have established a screening library that enabled us to probe the potential functions of 17 GNAT enzymes and showed 5 enzymes are inactive toward any of these compounds. Our results also showed specific structural moieties on polyamine and polyamine-like compounds are recognized by these enzymes. Finally, we have made this assay accessible to others interested in querying the potential functions of GNAT enzymes by providing detailed information about compounds, hazards, solubility, and other considerations in assay design and their limitations. Further structural and enzyme kinetic characterization of these enzymes is currently ongoing in our laboratory.

MATERIALS AND METHODS

Chemicals. A description of all acceptor substrates considered for assays and the corresponding solubility, quantity, chemical hazards, and chemical suppliers are listed in Tables S1 and S2.

Clones. All clones and their corresponding UniProtIDs and vectors are described in Table 2. For simplicity, we refer to internal protein IDs listed in this table throughout the text. The following proteins were cloned from their corresponding genomic DNA using previously described procedures:[40] 99010, 99015, 99020, 99021, 99025, 99029. The 99011, 99016, 99019, 99022, 99026, 99027, and hSSAT1 proteins were synthesized with optimized *E. coli* codon usage from Twist Biosciences. The S10 and S15 clones were generated as described previously and were kindly supplied by Jade Forwood at Charles Sturt University.[41] The VcSpeG and AAC(6′)-Ig clones are the same as described before.[11,28]

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Growth, Protein Expression, and Purification. The following proteins were purified at Argonne using previously described procedures:[42] 99010, 99015, 99020, 99021, 99025, and 99029. All other proteins (99011, 99016, 99019, 99022, 99026, 99027, S10, S15, AAC(6′)-Ig, hSSAT1, and VcSpeG) were purified at SFSU using previously described procedures.[43] The polyhistidine tag was removed from all proteins except AAC(6′)-Ig. All proteins were buffer exchanged into 10 mM Tris pH 8.3, 500 mM NaCl prior to enzymatic assays.

Enzyme Kinetics Assay. Assays were performed as described previously[5] with the following modifications. The 50 μL reaction contained 50 mM Tris HCl pH 8.0, 100 mM NaCl, 1 mM AcCoA, 1 mM acceptor substrate, and 1 μg of protein of interest. Reactions were incubated for 10 min at 37 °C and then stopped with 50 μL of solution (100 mM Tris HCl pH 8.0, 1 M guanidine HCl) and reacted with Ellman's reagent by adding 200 μL of solution (100 mM Tris HCl pH 8.0, 1 mM EDTA pH 8.0, 0.2 mM DTNB) to each well and incubated for 10 min at RT. The A_{415nm} was measured and the resulting OD after blank subtraction was converted to activity (μmol/(min·mg)) using the absorbance of L-cysteine standards to convert OD to nmol of CoA. Assays were performed in duplicate. The final list of 61 acceptor substrates screened for activity are listed in Table 1.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c08743.

Structures of all 61 compounds used in the final screening assay, identities of all compounds considered for the screening assay and their corresponding hazardous properties, and product information and solubility for the final 61 compounds used in the assay (PDF)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS AND SYMBOLS

(BAPP), 1,4-bis(3-aminopropyl)piperazine; (Dah), 1,7-diaminoheptane; (AcCoA), acetyl coenzyme A; (Agm), agmatine; (APMDS), aminopropyl(diethoxy)methylsilane; (Cad), cadaverine; (PPT), DL-phosphinothricin; (DTNB), 5,5′-dithiobis(2-nitrobenzoic acid); (GNATs), Gcn5-related *N*-acetyltransferases; (MSO), L-methionine sulfone; (MSX), L-methionine sulfoximine; (NAPPC), *N*-(3-aminopropyl)-2-pipecoline; (NAPPPR), *N*-(3-aminopropyl)piperidine; (PAPA), *N*-propyl-1,3-propanediamine; (232TA), *N*,*N*′-bis(2-aminoethyl)-1,3-propanediamine; (Nspd), norspermidine; (Spd), spermidine; (SSATs), spermidine/spermine *N*-acetyltransferases; (Spm), spermine; (TEPA), tetraethylenepentamine; (Tspm), thermospermine

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