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RESEARCH ARTICLE

Multisubstrate adduct inhibitors: Drug design and biological tools

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Abstract
In drug discovery, different methods exist to create new inhibitors possessing satisfactory biological activity. The multisubstrate adduct inhibitor (MAI) approach is one of these methods, which consists of a covalent combination between analogs of the substrate and the cofactor or of the multiple substrates used by the target enzyme. Adopted as the first line of investigation for many enzymes, this method has brought insights into the enzymatic mechanism, structure, and inhibitory requirements. In this review, the MAI approach, applied to different classes of enzyme, is reported from the point of view of biological activity.

Keywords: *Multisubstrate; bisubstrate; enzyme; inhibition; drug design*

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Introduction

One of the most important aspects of drug design is for small molecules to achieve high specificity and efficacy toward their given biological target, whether it is a membrane-bound receptor or an enzyme. For lead compound identification based on enzyme inhibition, several methods have been developed to enhance specificity and potency. Such compounds are transition-state analogs, suicide or mechanism-based inhibitors, and multisubstrate adduct inhibitors (MAIs)[1]. The design of transition-state analogs requires a precise understanding of the enzyme mechanism and of its transition-state/enzyme complex (E–S). Alternatively, suicide or mechanism-based inhibitors require, for inhibition, the molecule to interact with the target enzyme in such a way as to initiate a catalytic process, thus resulting in the formation of a stable inhibitor–enzyme complex. The MAI approach can potentially be applied to all enzymatic reactions in which at least two molecules (cofactor included) are simultaneously present and reacting in the enzyme active site. As such, the combination of structural features taken from each reagent into a single molecule potentially increases the binding efficacy as order is introduced, and the binding specificity due to the substrate/cofactor synergistic effect on recognition patterns. This combination of several substrates involved in an enzymatic reaction was termed by Wolfenden as multisubstrate[2]. Currently, the design of MAIs is one of the best means to obtain mechanistic and structural information on an enzyme and to create new inhibitors with potentially high potency.

Often the use of a cofactor mimic moiety allows for an increase in affinity toward the enzyme, while analogs of the substrate that compete with the natural substrate in the active site enhance the specificity. However, two important aspects in the MAI approach have to be considered: the points of attachment of the linker between the cofactor and the substrate and the length of this linker, which must allow for binding to both substrate and cofactor pockets. We will now present developments that have been made in drug design through the MAI approach by examining the different classes of enzyme and the approach to their inhibition.

1. Lyases

1.1. Ornithine decarboxylase

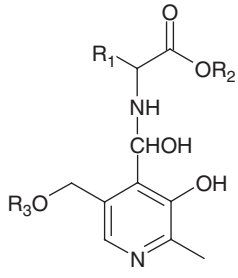
One of the first studies on MAIs described specific inhibitors for prokaryotic and eukaryotic ornithine decarboxylase (EC 4.1.1.17)[3]. Ornithine decarboxylase (ODC) catalyzes the decarboxylation of ornithine, producing the diamine putrescine. ODC utilizes pyridoxal-5′-phosphate (PLP) as the cofactor and ornithine or lysine as the substrate. Coward *et al.* synthesized the reduced Schiff base adduct analogous to the Schiff base involved in enzyme-catalyzed decarboxylation (Table 1).

The reduced Schiff base adducts 1a–c were tested as inhibitors of several decarboxylases and aminotransferases. It was found that adducts 1a and 1c were both efficient against ornithine and lysine decarboxylase. In the same manner, it was shown that apo-dopa decarboxylase (EC 4.1.1.28) was inhibited by both *N*-(5′-phosphopyridoxyl) tyrosine and *N*-(5′-phosphopyridoxyl)phenylalanine, while other PLP–amine adducts were much less effective.

1.2. Porphobilinogen synthase

Porphobilinogen synthase (PBGS) (5-aminolevulinate dehydratase (ALAD); EC 4.2.1.24) catalyzes the condensation of two molecules of 5-aminolevulinic acid (ALA; Scheme 1) to produce porphobilinogen (PBG), which is an intermediate in the biosynthesis of tetrapyrrolic natural products such

Table 1. Effect of adduct 1 at various concentrations of pyridoxal-5′-phosphate (PLP).



1 a $R_1 = (CH_2)_3NH_2$, R_2 = H, R_3 = P(O)(OH)$_2$
b $R_1 = (CH_2)_3NH_2$, $R_2 = R_3$ = H
c $R_1 = (CH_2)_4NH_2$, R_2 = H, R_3 = P(O)(OH)$_2$

Enzyme	[PLP] (mM)	[Amino acid] (mM)[a]	Inhibition[b] (%) 1a	1b	1c
ODC	0.075	0.64 (0.07)	90	0	85
LDC	0.05	3.0 (1.5)	75	0	90
SAMDC	0	0.1 (0.05)	0	0	nd
TAT	0.05	6.3	22	8	nd

Note. Enzymes used are abbreviated as follows: ODC, L-ornithine decarboxylase (EC 4.1.1.17); LDC, L-lysine decarboxylase (EC 4.1.1.18); SAMDC, *S*-adenosyl-L-methionine decarboxylase (EC 4.1.1.50); TAT, tyrosine aminotransferase (EC 2.6.1.5).

[a] K_m (mM) for amino acid given in parentheses.

[b] All inhibition present at a final concentration of 1.0 mM; nd, not determined.

as porphyrins and chlorophylls [4]. In an aim to increase the knowledge of the active site of the enzyme and to elucidate its mechanism, Neier *et al.* developed bisubstrate analogs (Scheme 1) which incorporated two γ-keto carboxylic groups for recognition with the enzyme's active site, and were attached together by several linkers[5].

These bisubstrates were evaluated as potential inhibitors of Mg^{2+}-dependent PBGS from *Pseudomonas aeruginosa* (ALA K_m = 0.33 mM). It appeared that bisubstrates with a small linker (a–c) were about 500-fold more active than were the two others. The poor affinity of the compounds d and e toward the enzyme could be explained by the size of the linkers sulfoxide and sulfone, which are bigger than amine or thio linkers[5].

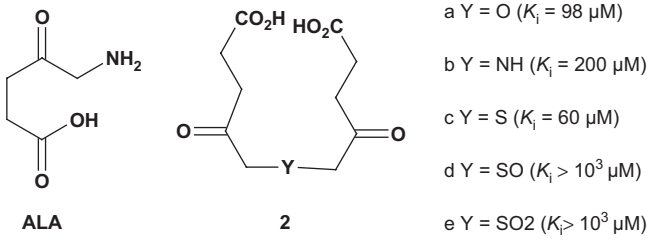
2. Ligase

2.1. Adenylosuccinate synthase

Adenylosuccinate synthase (AdSS; EC 6.3.4.4) catalyzes the first step in the transformation of inosine monophosphate (IMP) into adenosine monophosphate (AMP)[6]. Hydantocidin, known as pro-herbicide, is first converted into hydantocidin monophosphate (HMP), and this molecule mimics either IMP or AMP. Evidence from crystallographic studies of the enzyme showed that hydantocidin replaced IMP in the active site[7–10]. Hadacidin is also a natural substrate of AdSS, which was reported as a competitive inhibitor of aspartic acid but affecting the enzyme at a different site[11]. Based on the crystal structure of the complex AdSS–hadacidin, Hanessian *et al.* synthesized two bisubstrate hybrids composed of a covalent attachment between HMP and hadacidin (Scheme 2)[12].

These two adducts were evaluated as inhibitors of AdSS from *E. coli* and wheat (IMP K_m = 0.041 mM), with 3b displaying an IC_{50} of 0.043 μM for AdSS from *E. coli* and 0.200 μM for AdSS from wheat.

The inhibitor 3b was 10- and 100-fold more potent than HMP and hadacidin respectively. The linking of two relatively weak active inhibitors into a single molecule such as 3b enhanced significantly the enzymatic inhibitory activity as well as specificity when compared to the individual natural substrates[12].



Scheme 1. Bisubstrate analogs for porphobilinogen synthase.

2.2. Acetyl-CoA carboxylase

Acetyl-CoA carboxylase (ACC; EC 6.3.4.9) catalyzes the biotin-dependent carboxylation of acetyl-CoA to produce malonyl-CoA. ACC has been connected to obesity, type 2 diabetes, and microbial infection[13–16]. This makes ACC a target of choice for the development of new drugs. Based on a steady-state kinetic study, Waldrop synthesized and evaluated a unique bisubstrate analog as inhibitor of ACC (Scheme 3) featured by the coenzyme-A linked to the chloroacylated biotin analog via an acyl bridge[17,18].

Using malonyl-CoA (K_m = 0.1 mM) as a variable substrate, 4 showed competitive inhibition, with a K_i value of 23 ± 2 μM. In contrast, when biotycin (K_m = 8.25 mM) was applied as the variable substrate, non-competitive inhibition was observed[18].

2.3. Salicyl-AMP ligase

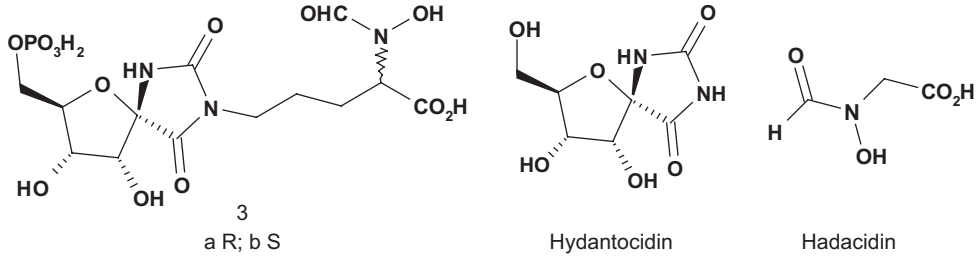
The elucidation of the mycobactrin biosynthetic pathway showed that salicyl-AMP (MbtA) is involved in the initiation of mycobactrin chain growth. Using this finding, Aldrich *et al.* developed new bisubstrate analog inhibitors of salicyl-AMP, with an acylsulfamate linkage mimicking the acyl-adenylate intermediate (Scheme 4)[19–25].

Biochemical analysis of MbtA using bisubstrate inhibitor has provided details of the reaction mechanism. With these results, Aldrich *et al.* provided information for future structure–activity relationship (SAR) studies and moreover were able to identify several modifications to the glycosyl inhibitors template, in particular on the nucleobase part, which improved the inhibitory activity[26]. Species specificity was also evidenced; quite noticeable is the identification of novel compounds as potent inhibitor (5b) of *M. tuberculosis* growth such as isoniazid, the most commonly prescribed anti-tuberculosis drug.

3. Oxidoreductase

3.1. Dopamine β*-hydroxylase*

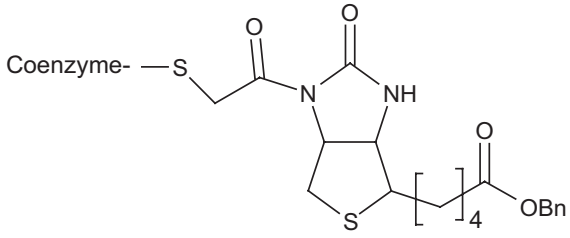
Dopamine β-hydroxylase (DβH; EC 1.14.17.1) is a tetrameric, copper containing, mixed-function oxidase that catalyzes benzylic hydroxylation of dopamine to (*R*)-norepinephrine



Scheme 2. Bisubstrate hybrids hydantocidin monophosphate (HMP)/hadacidin for adenylosuccinate synthase.

in the sympathetic nervous system[27]. Kruse *et al.* hypothesized that the DβH-catalyzed oxidation involved simultaneous binding of oxygen to a copper(I) site and to the phenylethylamine near the aromatic binding site. Thus, they developed new multisubstrate inhibitors (Scheme 5)[28]. In this first study, a phenyl or 4-oxygenated phenyl group was used as a mimic of the dopamine catecholic nucleus for recognition by the aromatic binding site of the enzyme. Synthesis and testing of a first panel of inhibitors was undertaken in order to optimize the space between the aryl system and the dopamine catechol mimic[28]. Compound 6 (X = OH, Y = CH_2, IC_{50} = 2.6 μM, K_i = 0.00549 μM at pH 4.5, 0.344 μM at pH 6.6) binds DβH approximately 10^5-fold more tightly than the tyramine (K_m = 5.65 mM) and appears more potent than furasic acid, the inhibitor which underwent clinical trials.

The same group reported a SAR study on 1-benzylimidazole-2-thione and its derivatives (Scheme 5)[29]. This allowed identification of the inhibitor's metabolic liability and determination of whether the affinity of multisubstrate inhibitors to DβH occurs by direct binding to the phenethylamine substrate site. Another series of derivatives were then prepared and some more potent inhibitors were revealed[30]. The most potent inhibitor (6: X = 3,5-F_2, 4-OH, Y = CH_2, IC_{50} = 0.074 μM) was binding the enzyme approximately 10^6-fold more tightly than tyramine. However, a lack of oral bioavailability was also identified, and this was associated with the phenolic group, which is a good site for metabolic conjugation *in vivo*. Fortunately, the 3,5-dihalo-substitution pattern was a viable replacement for the metabolically liable 4-hydroxyl group[29]. In order to improve the potency of the previous inhibitor, Berkowitz *et al.* reported a new approach with substituted pyridyl moieties as isostere of the aryl groups present in substrates such as phenethylamine and *p*-tyramine and in the 1-(arylmethyl)imidazole-2-thiones (Scheme 6)[31].



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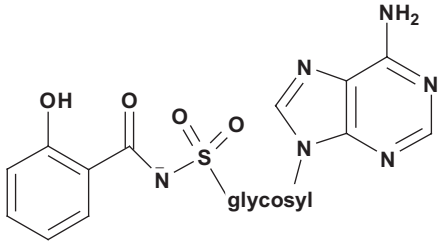
Scheme 3. Bisubstrate analogs for acetyl-CoA carboxylase.

The compounds 7a–c and 8, 9, 10 were tested for inhibition of DβH *in vitro* and *in vivo* (Table 2), with reference data for three 1-(arylmethyl)-imidazole-2-thiones[28–30] and a standard DβH inhibitor (furasic acid)[32]. Compounds 7a–c were potent as DβH inhibitors *in vitro* ($IC_{50} \approx 10^{-4}$ M) and even more potent *in vivo*. Particularly for compound 7a (2-pyridyl), the *in vivo* effects were comparable to those of furasic acid. Of the oxy-substituted compounds 8–10, only the 5-hydroxy-2-pyridylmethyl compound (8) was efficient against DβH ($IC_{50} \approx 10^{-5}$ M) *in vitro*, but unfortunately it had a weak antihypertensive effect *in vivo*.

The same group then investigated the possibility of enhancing inhibitor potency by modifying the copper-binding portion of the inhibitors in order to obtain further information about the enzyme copper binding site[33]. These results showed that the soft sulfur was clearly required for optimal activity. After having established the sulfur requirement, the investigation moved toward variation of the ligand ring. A series of DβH alkylaryl-substituted heterocycles were synthesized and tested for inhibition of DβH. The data showed weak affinities[34]. A chiral approach was reported for inhibition of DβH. The series of novel potent, rigid, and chiral bisubstrate inhibitors was



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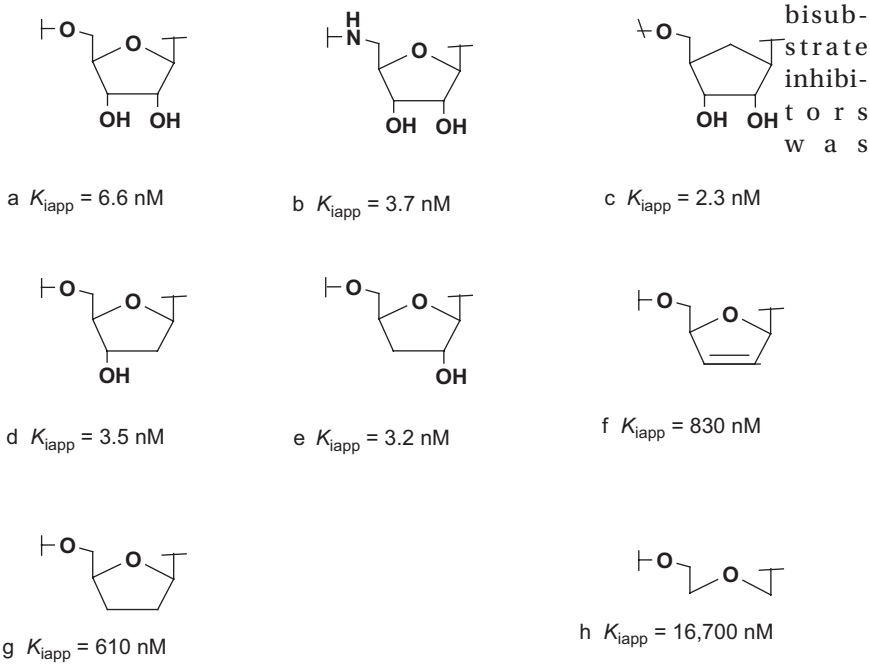
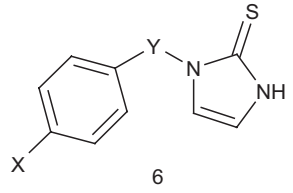


Scheme 4. Bisubstrate analogs for salicyl-AMP.

designed to mimic phenylethylamine and dioxygen substrates in order to study the electronic, steric, and proximity constraints of the DβH active site[27]. These compounds were tested, and the results indicated that the DβH active site possessed a steric and electronic tolerance toward these inhibitors, allowing for the interaction of the enantiomeric pairs of inhibitors with the enzyme especially in the region of the two copper centers. It should be emphasized that 11a and 11b (Scheme 7) were found to be potent competitive inhibitors of DβH.

In addition, the transposition of the oxygen and nitrogen in the thiooxazolidone ring of 11a and 11b by displacement of 5-phenyl to 4-phenyl (compounds 12a and 12b) decreased strongly the inhibition potency.



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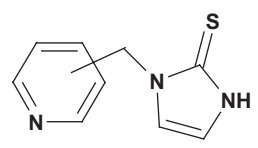
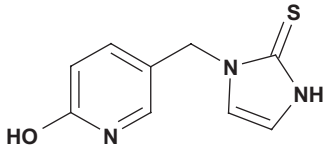
Scheme 5. Multisubstrate adduct for dopamine β-hydroxylase.



7 a–c



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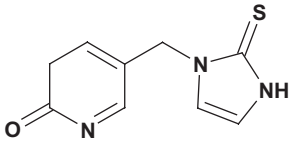

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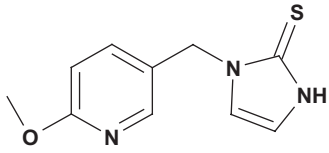

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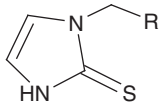
Scheme 6. Modified multisubstrate adducts for dopamine β-hydroxylase.

Table 2. DβH inhibition DA/NE ratio[a] and antihypertensive activities of 1-(pyridylmethyl)imidazole-2-thione.



No.	R	IC_{50} (μM)	Increase in DA/NE ratio (%)	Decrease in BP (mmHg)
7a	2-Pyridyl	131 (76–220)	141 ± 6	35 ± 14
7b	3-Pyridyl	104 (89–121)	52 ± 11	34 ± 11
7c	4-Pyridyl	17%[c]	49 ± 8	38 ± 15
8	5-Hydroxy-2-pyridyl	27 (21–35)		22 ± 17
9	2-Pyridon-5-yl	7%[c]		
10	2-Methoxy-pyridyl	17%[c]		
	4-OHC_6H_4	2.6 (1.3–4.6)	95 ± 15	22 ± 4 (n = 3)
1[b]	3,5-F_2-C_6H_3	1.2 (1.1–1.4)	407 ± 30	50 ± 4 (n = 3)
Furasic acid		0.7 (0.4–1.1)	80 ± 35	54 ± 7 (n = 4)

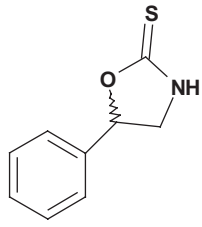
[a]DA/NE, dopamine/norepinephrine.
[b]Classification from publication.
[c]Represents percent inhibition observed at 10^{-4} M.

3.2. 4-Hydroxybenzoate 3-monooxygenase

4-Hydroxybenzoate 3-monooxygenase (PHBH; EC 1.14.13.2) is well known as the NADPH-dependent enzyme flavin-monooxygenase. The enzyme mechanism has been characterized as a random sequential addition of the two substrates *p*-hydroxybenzoic acid (K_m = 21 μM) and NADPH (K_m = 57 μM) to the enzyme and oxidized flavin cofactor[34].

Salituro *et al.* reported the design of new multisubstrate inhibitors in which the NADPH mimic was simplified by an unsubstituted phenyl ring, to stabilize the π–π interaction between the aromatic ring of this group and the flavin nucleus[34]. In addition, a methyleneoxy moiety was used as a linker between the *p*-hydrobenzoic acid and the NADPH mimic (Scheme 8).

Both potential inhibitors were initially evaluated by modeling experiments, which showed that 13 was stacked under the flavin's B ring with a similar position to the *p*-hydrobenzoate substrate, whereas 14 moved out of the plane. This suggested that 13 would be the best potential inhibitor due to the favorable π–π interactions[34]. Then, both compounds were tested as inhibitors of PHBH and, in agreement with the modeling predictions, 13 had the highest affinity (K_i = 59 nM) for the enzyme.



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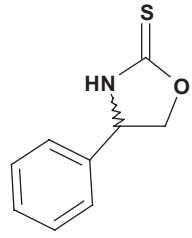
a R K_i = 4.1 μM

b S, c RS K_i = 13.7 μM



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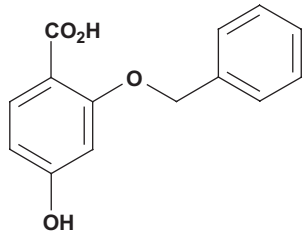
a R K_i = 47 μM

b S K_i = 142 μM

Scheme 7. Rigid and chiral bisubstrates of dopamine β-hydroxylase.



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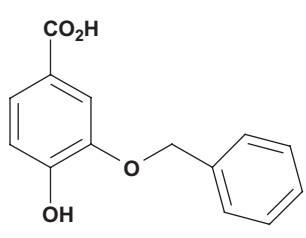

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Scheme 8. Multisubstrates for 4-hydroxybenzoate 3-monooxygenase.

4. Transferases

4.1. 3-Deoxy-D-manno-2-octulosonate-8-phosphate (KDO8P) synthase

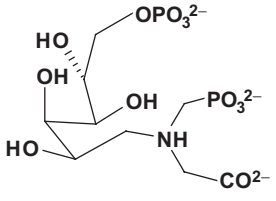
KDO8P synthase (EC 2.5.1.55) catalyzes the condensation reaction between D-arabinose-5-phosphate (A5P) and phosphoenolpyruvate (PEP), forming KDO8P and releasing an inorganic phosphate[35].

Du *et al.* reported the synthesis of the first bisubstrate inhibitor of KDO8P synthase, which is crucial in the assembly process of the lipopolysaccharides of most gram-negative bacteria[36–38]. According to studies carried out on the enzyme's mechanism, A5P and an amino PEP analog could be combined (Scheme 9) to give access to 15[39–43].

Compound 15 was evaluated as an inhibitor of KDO8P synthase to obtain insights into the enzyme mechanism. Bisubstrate 15 appeared to bind the enzyme 1500-fold more tightly than KDO8P and 20-fold more tightly than PEP[36]. The study showed that 15 functioned as a slow-binding inhibitor with a dissociation constant of 0.42 μM (K_m (PEP) = 8 μM).

4.2. Spermidine synthase and spermine transferase

The biosynthesis of polyamines is carried out by three highly conserved polyamine biosynthetic enzymes: ornithine decarboxylase, putrescine aminopropyltransferase (PAPT), and spermidine aminopropyltransferase (SAPT). Inhibition of polyamine biosynthesis has potential as chemotherapy and as antiproliferative therapy[44,45]. A series of aminopropyltransferase inhibitors have been developed for spermidine synthase (PAPT; EC 2.5.1.16) and spermine transferase (SAPT; EC 2.5.1.22)[46]. It has been demonstrated, in the case of spermidine synthase from *E. coli*, that transfer of the aminopropyl group occurs via a ternary complex by direct nucleophilic attack[47]. Synthesis and biological evaluation of *S*-adenosyl-1,8-diamino-3-thiooctane (AdoDATO; 16a) showed that this compound was the most potent inhibitor known for spermidine synthase[46,47]. This high potency of inhibition was due to the fact that AdoDATO was a multisubstrate analog of the transition state[48]. From this preliminary study, *S*-adenosyl-1,12-diamino-3-thio-9-azadodecane (AdoDATAD; 16b) was synthesized and biologically evaluated as the corresponding multisubstrate adduct inhibitor for the spermine synthase reaction. AdoDATO provided the structural basis for the design of 3-(*R,S*)-(5′-deoxy-5′-carbadenos-6′-yl) spermidine (AdoSpd; 16c). This inhibitor displayed good inhibition of putrescine aminopropyltransferase (PAPT)[49]. Nevertheless, AdoSpd was less selective than AdoDATO, and inhibited spermidine aminopropyltransferase (SAPT) to a significant extent (Scheme 10).



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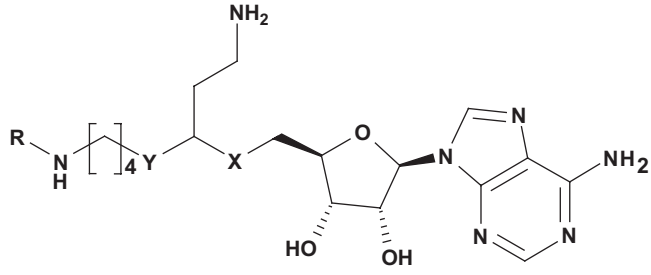
Scheme 9. Bisubstrate for 3-deoxy-D-manno-2-octulosonate-8-phosphate (KDO8P) synthase.

4.3. Glycinamide ribotide transformylase, 5-amino-4-imidazol carboxyamide ribotide transformylase, and thymidylate synthase

4.3.1. Glycinamide ribotide transformylase

Glycinamide ribotide transformylase (GAR-Tase; EC 2.1.2.2) catalyzes the first step in the *de novo* purine biosynthesis pathway requiring folate as a cofactor[50]. A physiological effect was obtained with 5,10-dideaza-5,6,7,8-tetrahydrofolic acid against solid tumor and this was attributed to the inhibition of GAR-Tase[51]. Benkovic *et al.* described the first successful MAI (β-TDDG) for GAR-Tase with a very good affinity for the enzyme, with a K_i value three-fold higher than that of the substrate (K_m (GAR) = 23 μM) (Scheme 11)[52]. β-TDDG acted as a slow, tight-binding inhibitor against four species of GAR-Tase (*E. coli*, Avian, Hela O, and L1210). In addition, β-TDDG was the most potent inhibitor reported for this enzyme: K_d = 250 ± 50 nM. Using this compound, Wilson solved the crystal structure of the GAR-Tase active site[53–55].

4.3.2. 5-Amino-4-imidazol carboxyamide ribotide transformylase (AICAR-Tase)



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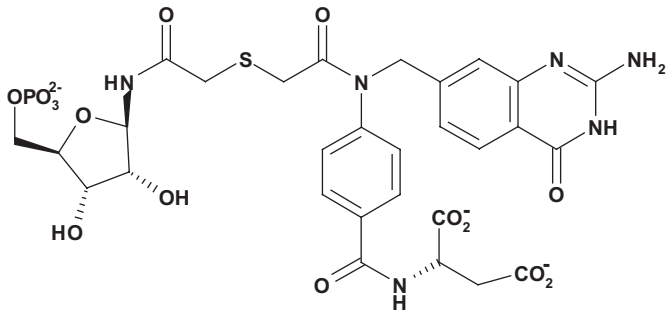
a R = H, Y = CH_2, X = S;
b R = $NH2(CH_2)3$, Y = CH_2, X = S;
c R = H, Y = NH, X = CH_2

Scheme 10. Multisubstrate adducts for spermine synthase reaction.



17

β-TDDG

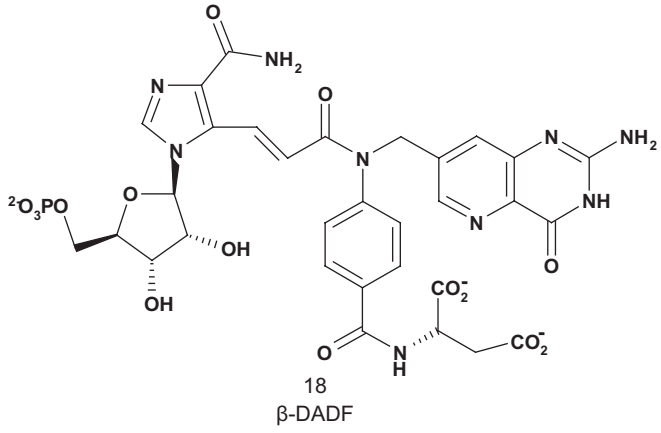
Scheme 11. Multisubstrate for glycinamide ribotide transformylase (GAR-Tase).

Benkovic also developed the first MAI for AICAR-Tase (EC 2.1.2.3), β-DADF (18) (Scheme 12), including both substrates used by the enzyme[56]. β-DADF was evaluated as an inhibitor of AICAR-Tase, with a K_d of 20 nM. β-DADF was bound 10^3-fold more tightly than each of the other substrates by the transferase, with complete selectivity for the AICAR enzyme over GAR-Tase[57].

4.3.3. Thymidylate synthase

The concept of "thymineless death" has for many years been used to justify thymidylate synthase inhibition (TS; EC 2.1.1.45) as a target for cancer chemotherapy, as demonstrated by Koyama *et al.*[58–60].

Mertes *et al.* synthesized the first thymidylate synthase inhibitor with a K_i of 0.75 μM, which was a thymidylate substituted on the 5-methyl with a simple tetrahydroquinoxaline[61]. Subsequent to this, Broom *et al.* described the synthesis and biological evaluation of other multisubstrate analog inhibitors (Table 3)[59].



18
β-DADF

Scheme 12. Multisubstrate for amino-4-imidazol carboxyamide ribotide transformylase (AICAR-Tase).

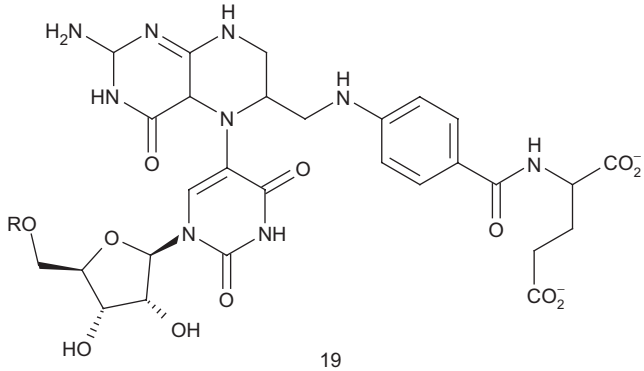
Compound 19b proved to be a potent competitive inhibitor of TS. Broom *et al.* then focused on the synthesis of bisubstrate analogs that would retain the binding abilities of 19b and have greater flexibility, but without the chiral carbon[62]. Unfortunately, these new bisubstrates were less potent inhibitors than 19b. The hypothesis was that the 6-position of the pyrimidine might undergo attack by a sulfhydryl group present in the active site in order to create a ternary complex resembling 19b. This last study suggested that these inhibitors fitted the active site, but might act more as product-substrate analogs than as -bisubstrate analogs[62].

Recently, Lebioda *et al.* reported cooperative inhibition of hTS exploiting both active site and allosteric inhibitions, creating synergy by using two inhibitors binding two different subunits of TS[62]. The concept developed here to target two non-equivalent sites may alleviate the development of drug resistance in patients, even though the resistance through increased expression cannot yet be addressed by this approach.

4.4. Protein kinases

Kinases catalyze the transfer of the γ-phosphate of nucleoside triphosphates (usually adenosine triphosphate; ATP) to a functional group on an acceptor molecule. X-ray crystallography and nuclear magnetic resonance (NMR) analysis have been particularly useful in helping to define the nature of the binding sites[64]. The progress in this field has shown that such inhibitors could be invaluable as biological reagents and serve as therapeutically useful compounds for the treatment of a wide variety of diseases[64].

Table 3. Inhibition of human thymidylate synthase.



19
a R = H; b R = PO_3^{2-}; c R = CH_3PO_3–

	Variable (dUMP)				Variable ($N^{5,10}$-CH_2-H_4PteGlu)			
Compound	Inhibition type	K_i (μM)	K_i/K_m	K_i/K_i (19b)	Inhibition type	K_i (μM)	K_i/K_m	K_i/K_i (19b)
dUMP		3.0		50				
19b	C[a]	0.058	0.02	1.0	C	0.25	0.005	1.0
19c	C	120	40	2068	NC	700	14	2800
19a	NC	150	50	2586	NC	240	4.8	960
$N^{5,10}$-CH_2-H_4PteGlu						50		200

[a]C, competitive; NC, non-competitive.

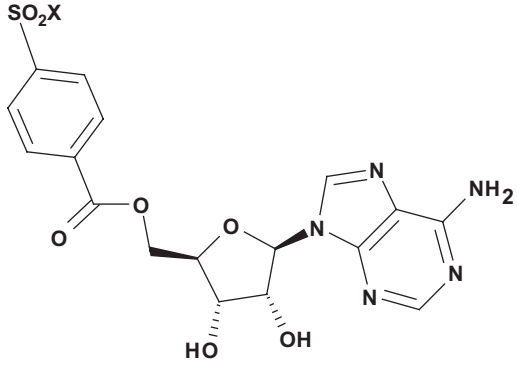
4.4.1. Tyrosine kinase

According to Holden, the MAI approach for protein kinases (PKs) is only useful with enzymes that directly phosphorylate their substrate without formation of a phosphorylated enzyme intermediate. As a result of studies into other classes of kinases and Goldberg's and Wong's investigations, it is now possible to assume that tyrosine phosphorylation of angiotensin II proceeds via a ternary complex, thus amenable to inhibition by multisubstrate analogs[65].

Based on this, Holden *et al.* adopted the MAI approach to probe the distance between the ATP and tyrosine binding sites in a tyrosine-specific protein kinase (Scheme 13)[66].

Poor inhibition was achieved with compound 20, and as such they subsequently reported the synthesis and evaluation of multisubstrate inhibitors containing a polyphosphate linkage between the tyrosine mimic block and the adenosine (Table 4)[66]. However, the improvement of inhibitory activity was not really significant.

Traxler *et al.* considered the multisubstrate inhibitor approach to develop potential bisubstrate inhibitors of the epidermal growth factor (EGF)-receptor tyrosine kinase[67]. Their strategy was to combine both elements of ATP and tyrosine. Therefore, 5′-[4-(fluorosulfonyl)benzoyl]adenosine (5′-FSBA) was covalently attached to tyrosine mimics via a sulfonyl moiety (Scheme 14)[68–71]. Compounds were tested as selective inhibitors of EGF-receptor (EGF-R) tyrosine. Compounds 23–24 showed a moderate inhibitory activity for EGF-R tyrosine kinase. Their IC_{50} values were similar to that of erbstatin[72]. However, these compounds were selective for EGF-R tyrosine kinase. A stronger inhibition was observed for compounds 22a–s, with K_i values of about 1 μM. The most potent inhibitor (R_1 = H; R_2 = H; R_3 = 2-OH; R_4 = OH) showed a K_i value of 0.054 μM. Additionally, it offered high selectivity for EGF-R tyrosine kinase with respect to v-abl tyrosine kinase and protein kinase C (PKC)[67].



20

Scheme 13. Multisubstrate for tyrosine kinase.

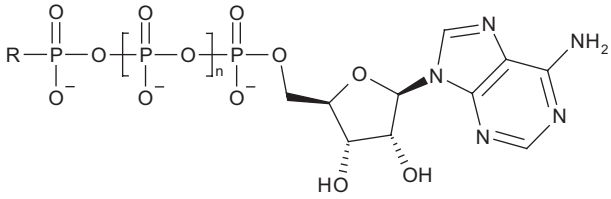
Recent investigations into the design of inhibitors for PKs led Uri's group to develop nanomolar bisubstrate analog inhibitors of basophilic PKs[64]. Their strategy aimed to combine an oligoarginine peptide with adenosine, adenosine-5′-carboxylic acid, and 5-isoquinolinesulfonic acid (Scheme 15).

All these compounds have been evaluated as inhibitors of cAPK (calmodulin-dependent protein kinase), and 25d and 25b were found to be the most potent inhibitors with an IC_{50} of 8.3 nM and 5.3 nM, respectively.

4.4.2. Adenylate kinase

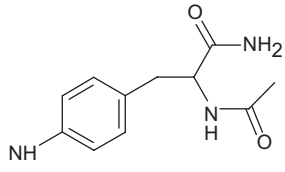
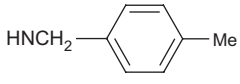
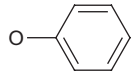
Adenylate kinase (AK; EC 2.7.4.3) was first investigated with the MAI approach by Wolfenden and Lienhard. The latter used this strategy to develop a potent inhibitor (Scheme 16) of AK[73]. This enzyme is a phosphotransferase enzyme that catalyzes the reversible transfer of the terminal phosphate group between ATP-Mg^{2+} and adenosine monophosphate (AMP) to provide two adenosine diphosphates (ADPs)[74].

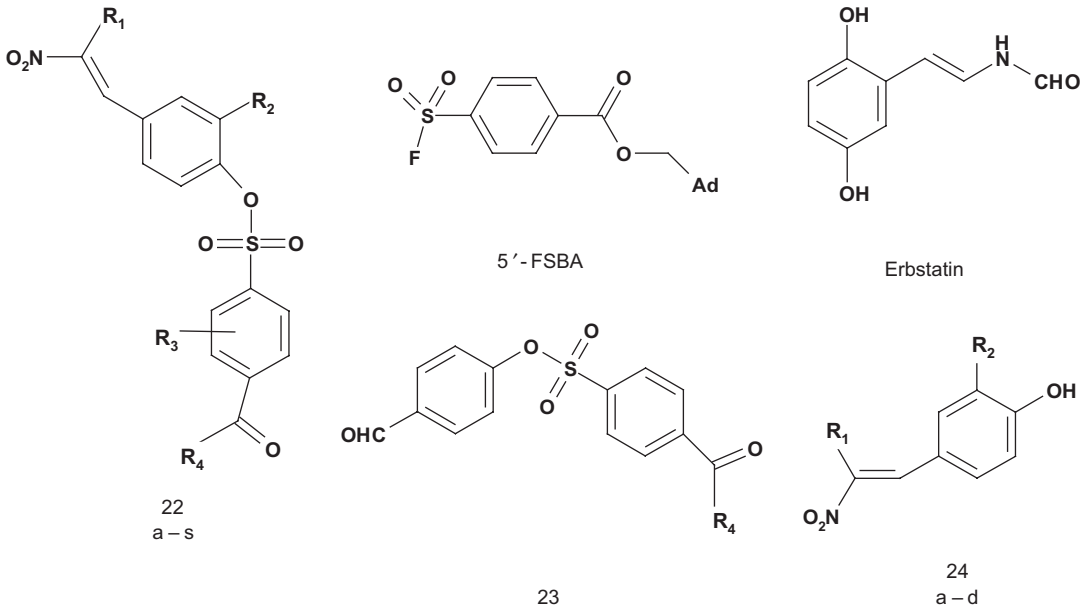
Table 4. Inhibition of tyrosine kinase.



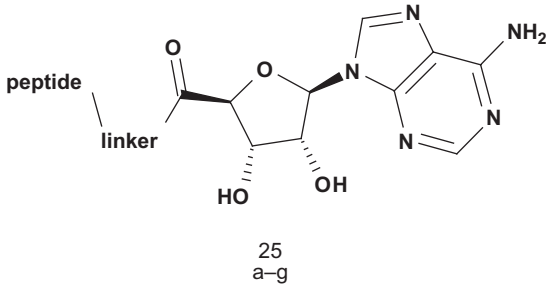
21
a–h

21a–h

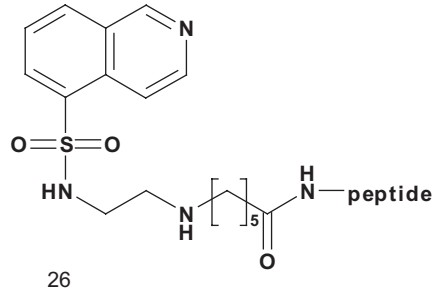
Compound	R	*n*	IC_{50} (M)	Compound	R	*n*	IC_{50} (M)
21a		1	150	21e		1	44
21b		2	16	21f		2	110
21c		1	90	21g		1	13
21d	$NHCH_2CH_2NHC(O)Me$	1	25	21h		2	41



Scheme 14. Bisubstrates for epidermal growth factor (EGF)-receptor tyrosine kinase.



Linker	Peptide
a $NH(CH_2)_5CO$	$(L\text{-}Arg)_4NH_2$
b $NH(CH_2)_5CO$	$(L\text{-}Arg)_6NH_2$
c $NH(CH_2)_5CO$	$(D\text{-}Arg)_4NH_2$
d $NH(CH_2)_5CO$	$(D\text{-}Arg)_6NH_2$
e $N(CH_3)(CH_2)_5CO$	$(L\text{-}Arg)_4NH_2$
f $NHCH_2$-Ph-4-CO	$(L\text{-}Arg)_4NH_2$
g NH-Ph-β-Aln-CO	$(L\text{-}Arg)_4NH_2$

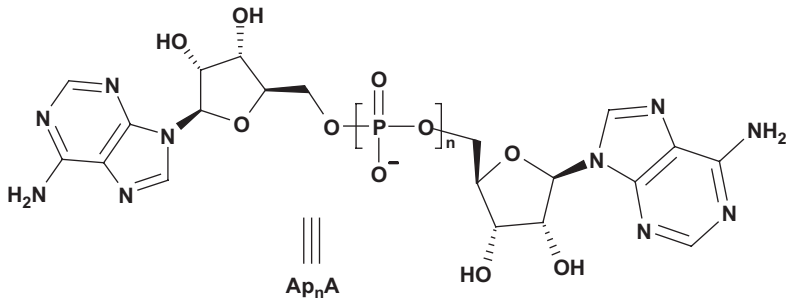


a peptide = $NH(CH_2)_3NHC(O)(CH_2)_2C(O)NH(L\text{-}Arg)_4NH_2$

b peptide = $CH_2C(O)NH(CH_2)_3NHC(O)(CH_2)_2C(O)NH(L\text{-}Arg)_4NH_2$

Scheme 15. Bisubstrate analogs for basophilic protein kinases (PKs).

Thus, Lienhard synthesized the first series of MAIs ($n = 2$, 3, 4, 5), and their biological evaluation led to the identification of a potent inhibitor of adenylate kinase, Ap_5A, which was able to inhibit the reaction of adenylate kinase by 55% at 3×10^{-8} M. At the same time, Lienhard demonstrated that requirements for potent inhibition were that two adenosine groups needed to be linked by a polyphosphate bridge containing at least five phosphoryl groups[73].



27

Scheme 16. Multisubstrate for adenylate kinase.

4.4.3. Thymidine kinase and thymidylate kinase

Thymidine kinase (TK; EC 2.7.1.21) plays a central role in the nucleotide salvage pathway. It catalyzes the transfer of the γ-phosphoryl group of ATP to thymidine to produce thymidine monophosphate (TMP). It is an essential enzyme for cell proliferation, and thus an attractive target for the development of drugs against cancer. Thymidylate kinase (TMK) is also involved in activation of the acquired immunodeficiency syndrome (AIDS) prodrug azidothymidine (AZT)[75].

Wolfenden developed a series of inhibitors of TK and TMK based on Lienhard's finding, using a polyphosphate bridge to link adenosine and thymidine as a bisubstrate (Ap_ndT). In a first study, Wolfenden evaluated these molecules as inhibitors of TK, trying to increase the affinity to either cytosolic enzyme or mitochondrial enzyme[76]. The polyphosphate bridge featuring five phosphate groups was required for the greatest inhibition (11% and 18% of activity at 1.0 μM), which provided information on the distance between both binding sites. Wolfenden also reported the biological evaluation of Ap_4dT, Ap_5dT, Ap_6dT, Ap_4A, and Ap_6A as inhibitors of TMK[77]. Thymidylate kinase was inhibited by Ap_4dT, Ap_5dT, and most strongly by Ap_6dT (K_i = 0.20 μM ATP, K_i = 0.18 μM TMP). This last compound showed competitive inhibition when either TMP (K_m = 40 μM) or ATP (K_m = 0.25 μM) was varied. Ap_5dT was subsequently used by Reinstein *et al.* to solve the structure of TMK[75].

4.4.4. Deoxynucleoside kinases

This family consists of various deoxynucleoside kinases including cytidine (EC 2.7.1.74), guanosine (EC 2.7.1.113), adenosine (EC 2.7.1.76), and thymidine kinase (EC 2.7.1.21), which also phosphorylates deoxyuridine and deoxycytosine. These enzymes catalyze the production of deoxynucleotide 5′-monophosphate from a deoxynucleoside, using ATP as co-substrate.

Ives *et al.* used Lienhard and Wolfenden's proposal to evaluate a series of multisubstrate adducts as inhibitors of deoxynucleoside kinases. The molecules were structurally similar to the one being studied for TK, TMK, and AK, but one of two adenosines in the multisubstrate was deoxygenated on the 3′-position (dAp_nA). dAp_3A had the exact feature of the substrates for dAdo kinase. Unfortunately, it was found to be a weak inhibitor of dAdo kinase I, with a calculated K_{iapp} of 28 μM against ATP at a fixed dAdo concentration of 0.01 mM. In contrast, dAp_5A, with one more phosphate group, appeared to be a potent and specific inhibitor for dAdo kinases, with a K_{iapp} of 2.7 μM for dAdo kinase I[78].

4.4.5. Creatine kinase

Creatine kinase (CK; EC 2.7.3.2) catalyzes the synthesis of phosphocreatine (PCr), which is subsequently used in the regeneration of ATP in cell types where the consumption of ATP is rapid[79]. Steghens *et al.* reported the first synthesis and evaluation of bisubstrate inhibitors of CK combining creatine analog and hydrophobic moieties (Scheme 17)[80].

The evaluation was carried out at pH 6.6 and 8.6 for the two isoforms of CK, CK-MM and CK-BB. It appeared that at pH 6.6 there was competitive inhibition for CK-MM with DPPG versus both substrates CP and ADP, with K_{iapp} values of 28 μM and 1.3 μM, respectively. For CK-BB non-competitive inhibition was observed at this pH. However, at pH 8.6, competitive inhibition was detected for both enzymes CK-BB and CK-MM[80].

4.4.6. Phosphoglycerate kinase

Phosphoglycerate kinase (PGK; EC 2.7.2.3) is a transferase which catalyzes the interconversion of 1,3-*bis*-phosphoglycerate (1,3-BPG) and adenosine diphosphate with 3-phosphoglycerate (3-PGA) and adenosine triphosphate[81]. According to Blackburn, ideal analogs should feature five negative charges in the active site in addition to two on the glycerate 3-phosphate ester, and with a linker stable to hydrolysis[81]. Therefore, Blackburn *et al.* developed a chemical synthesis and determined binding characteristics for several non-hydrolyzable bisphosphonate analogs of 1,3-BPG[81–83]. The best ligand of the enzyme was compound 29, with a K_d value of about 1 μM (Scheme 18).

A decrease of the binding activity was also observed when the polyphosphate linker was composed of two phosphonates. This suggested that the affinity of bisubstrate analogs for PGK is dominated by coulombic interaction of the phosphoryl moiety[81].

4.4.7. 6-Hydroxymethyl-7,8-dihydropterin pyrophosphokinase

6-Hydroxymethyl-7,8-dihydropterin pyrophosphokinase (HPPK; EC 2.7.6.3) catalyzes the transfer of pyrophosphate from ATP to 6-hydroxymethyl-7,8-dihydropterin (HP), leading to the biosynthesis of folate cofactors. This is the first reaction in the folate biosynthetic pathway[84]. In contrast with mammals, microorganisms synthesize folates *de novo*, and therefore, as with all other enzymes in the folate pathway, HPPK is an interesting target for the development of an antimicrobial agent. Yan *et al.* synthesized three bisubstrate analogs each containing a pterin ring, an adenosine moiety, and a polyphosphate bridge as linker (Scheme 19)[85].

These three bisubstrates were biologically evaluated as potential inhibitors of HPPK, and it was shown that bisubstrate 30c ($n = 4$, $IC_{50} = 0.44$ μM) was the most potent inhibitor. The authors subsequently used 30c to determine the crystal structure of HPPK.

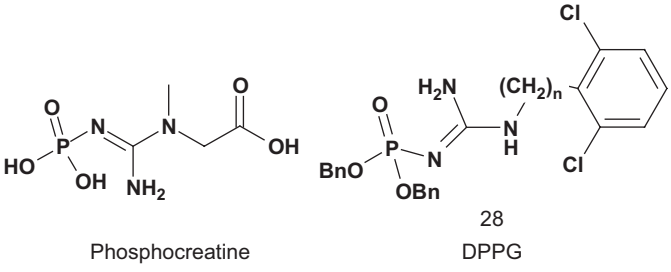
4.4.8. Protein kinases A, C, and IRK

Activators of protein kinase C (PKC; EC 2.7.11.13) such as phospholipids or diacylglycerol interact with the regulator domain, while both ATP and the protein substrate interact with the catalytic domain[86].

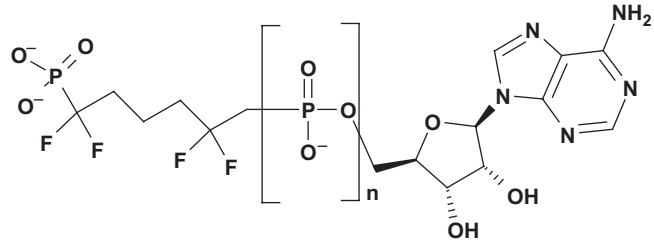
Sergheraert *et al.* reported the design of new bisubstrates as inhibitors of PKC which interact simultaneously with both regulator and catalytic domains[87]. These bisubstrates featured a cluster of arginine residues as substrate mimics, [(dimethyl-amino)naphthalenyl]sufonyl (dansyl) and 5-isoquinolinylsulfonyl groups as ATP mimics, and the spacer was constituted of four β-alanine residues. The inhibitory activities were measured on PKC and PKA (cAMP-dependent PK; EC 2.7.11.1) with histone as substrate. The most potent inhibitor was compound 31 (Scheme 20), with K_i values of 0.1 μM and 0.004 μM against PKC and PKA, respectively. Compound 31 was about 60-fold more active than 1-(5-isoquinoline sulfonyl)-2-methylpiperazine toward PKC and 750-fold more toward PKA[88].

Based on the previous studies, Cole *et al.* reported the effect of introducing an acetyl spacer[89]. In designing the bisubstrates, they selected aminoalanine as the serine mimic linked to ATPγS via an acetyl group, with kemptide used as peptide substrate[90,91].

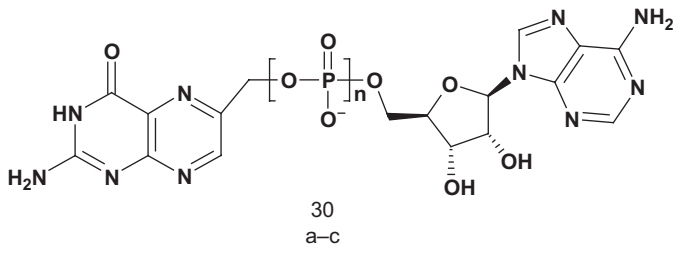
The evaluation was performed on the recombinant PKA expressed from *E. coli*, and it was found that 32 was a competitive inhibitor versus ATP ($K_m = 14.1$ μM), with a K_i value of 3.8 μM (Scheme 21).



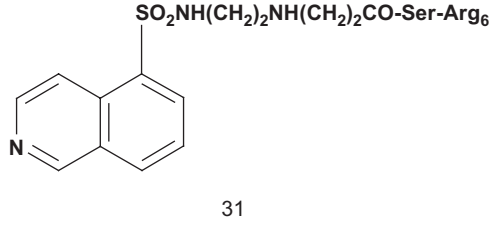
Scheme 17. Bisubstrates for creatine kinase.



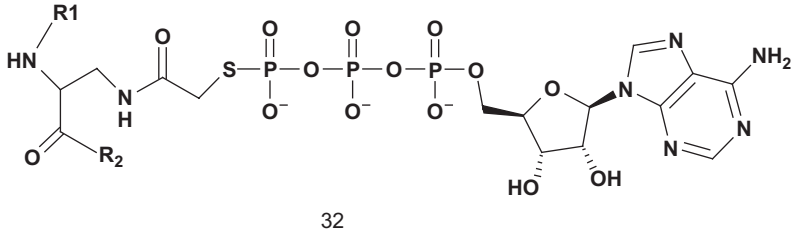
Scheme 18. Ligand of phosphoglycerate kinase.



Scheme 19. Bisubstrate analogs for 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase (HPPK).



Scheme 20. Bisubstrate analogs for protein kinase C.



Scheme 21. Bisubstrate analogs for protein kinase (1).

The bisubstrate analog described here was found to be more than 20-fold selective versus the related protein kinase, protein kinase C, and the less similar protein tyrosine kinase, Csk. This information highlighted the ability of the peptide moiety to contribute toward increased affinity and specificity of inhibition[89].

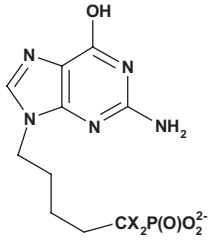
Cole *et al.* also investigated a new approach in bisubstrate design[92]. They based their study in part on the mechanism and structural consideration of the predicted dissociative transition state for PK, whereby they replaced the oxygen of tyrosine with an amino group that could be used as a hydrogen bond donor (Scheme 22).

Kinase assays with compound 33 revealed it to be a potent inhibitor of insulin-receptor kinase (IRK). It was a competitive inhibitor with regard to both nucleotide and substrate, with a K_i value of 370 nM, which was 190–760-fold lower than K_m values of the substrates[92].

4.5. Purine nucleoside phosphorylase

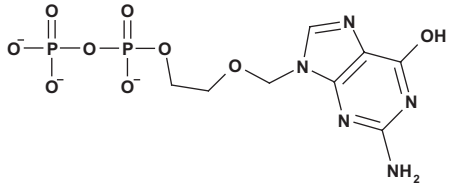
Purine nucleoside phosphorylase (PNPase; EC 2.4.2.1) catalyzes the reversible phosphorolysis of purine nucleosides such as inosine, 2′-deoxyinosine, guanosine, and 2′-deoxyguanosine to the purine and α-ribose or 2-deoxy-α-ribose 1-phosphate[93].

Table 5. Comparison of inhibitor constants of 34a and 34b for purine nucleoside phosphorylase (PNPase) from various sources.



34

a X = H; b X = F



35

PNP source	K_i (nM) 34a	34b	K_i 34a/K_i 34b
Human erythrocyte	320 ± 15	18 ± 3	18
Rat erythrocyte	905 ± 40	35 ± 2	26
Calf spleen	400 ± 80	16 ± 2	26
Escherichia coli	82 ± 5	15 ± 2	5.5

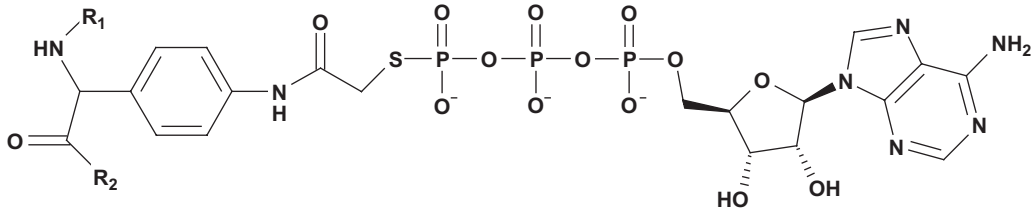
Inhibitors of human and parasitic PNPase are considered potential immunodepressive and anti-parasitic agents[94]. They may also have utility in the treatment of human T-cell leukemia, autoimmune disorders, and in the prevention of transplant rejection[95,96].

The diphosphate derivative of acyclovir (35) was a very potent inhibitor of the human enzyme (K_i = 8.7 nM, inosine K_m = 40 μM, 2′-deoxyinosine K_m = 65 μM, guanosine K_m = 46 μM)[97,98]. It is a metabolically stable "multisubstrate" acyclic nucleotide analog containing a purine and a phosphate-like moiety such as 9-phosphonoalkyl derivative. The most potent inhibitor of human erythrocytic PNPase in this series was 9-(5-phosphonopentyl)guanine (34a), but its K_i value was only 170 nM[99]. Later, Danzin reported the synthesis and the biological evaluation (Table 5) of the fluoro derivative 34b (9-(5,5-difluoro-5-phosphonopentyl)guanine), the analog of the phosphonate 34a.

These data were the first evidence of the superiority of a difluorophosphonate compound (34b) over a phosphonate (34a) as enzyme inhibitor. Later, Tuttle *et al.* based their strategy on Ealick's X-ray crystallography studies to enhance the potency of 9-phosphonoalkylguanine[101]. The hypothesis was to incorporate, into the 9-phosphonoalkylguanine, the appropriate spaced aryl substituent with affinity for the phosphate-binding site (Scheme 23)[102].

These multisubstrates 36 were tested for inhibition of human erythrocyte PNPase via a xanthine oxidase coupled assay with inosine as variable substrate. From this series, compounds 36 with X = 3-CH_2-OCH_2 (K_i = 5.8 nM) and X= 3-CH_2SCH_2 (K_i = 1.1 nM) were among the most potent inhibitors of PNPase reported[102]. After this study, Tuttle *et al.* envisaged synthesizing a stable mimic of the diphosphate 37a in which the oxygenated side chains were replaced by methylene moieties, as for (phosphinicomethyl)phosphate acid 37b (Scheme 24)[93].

The compounds were tested for inhibition of human erythrocyte PNPase. Two new potent inhibitors 37b, n = 5 and n = 6, which were stable mimics of 37a, were identified. They had K_i (inosine as variable substrate) in the nanomolar range when assayed in the presence of zinc chloride[94]. From this study, a model assuming interacting binding sites was more probable than the hypothesized dependent sites model.



33

R_1 = AcNH-Lys-Lys-Lys-Leu-Pro-Ala-Thr-Gly-Asp; R_2 = Met-Asn-Met-Ser-Pro-Val-Gly-Asp-CO_2H

Scheme 22. Bisubstrate analogs for protein kinase (2).

4.6. Methyl transferases

4.6.1. Catechol O-methyltransferase

Many enzymes catalyze transmethylation using *S*-adenosylmethionine (AdoMet or SAM), and catechol *O*-methyltransferase (COMT; EC 2.1.1.6) is one of them. Inhibition of COMT is considered an important approach in the development of new therapeutic treatments of Parkinson's disease, and recently this enzyme has been implicated in the modulation of pain[103,104].

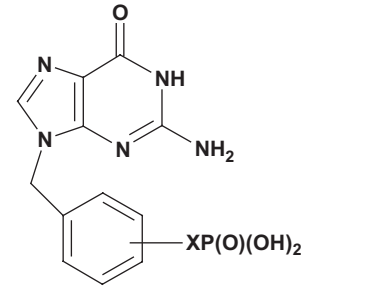
In a first study, Coward reported the synthesis and evaluation of novel potential multisubstrate inhibitors of COMT[105]. The synthesis was directed toward compound 38, which was assumed to be a potential transition-state analog.

Therefore, a series of compounds, 38, analogs of 34 and incorporating either a homocysteine or an adenosyl moiety and a sulfonium center, were synthesized (Scheme 25), and were shown to have satisfactory inhibitory activities with a K_i of approximately 1 mM[106]. Coward then designed other potential multisubstrate inhibitors of COMT and phenethanolamine *N*-methyltransferase (PNMT; EC 2.1.128). Unfortunately, the compounds had only weak inhibitory activity against COMT, and none of them were inhibitors of PNMT in assays using β-phenethanol-amine and AdoMet as substrates. Even though two inhibitors of COMT have now reached the market, there is to date no lead structure for bisubstrate inhibitors of this enzyme[107]. Therefore, Diederich *et al.* described a rational design, synthesis, and evaluation of new bisubstrate inhibitors of COMT (Scheme 26)[108]. They based their research on determination of the crystal structure of COMT complexed with SAM, 3,5-dinitrocatechol, and magnesium ion[109].

These compounds were tested as inhibitors of COMT isolated from rat liver. The results showed 39a as the most potent inhibitor (IC_{50} = 2 μM with preincubation, 4 μM without preincubation) as it was 10-fold more potent than 42b (IC_{50} = 25 μM with preincubation, 26 μM without preincubation). This behavior could be explained by the nature of the substrate, which allowed 39a to reach both catechol and SAM pockets. Compounds 42a (IC_{50} = 26 μM with preincubation, 25 μM without preincubation) and 42b (IC_{50} = 35 μM with preincubation, 27 μM without preincubation) had similar inhibitory activities, indicating that addition of the ribose component did not improve on the inhibitory effect of the catechol moiety[108].

Only recently has the first effective bisubstrate COMT inhibitor been developed, and for this type of compound they used both the nucleoside and catechol moieties to bind the SAM and substrate enzyme binding sites[110,111]. This work was extended by synthesizing a new series of bisubstrate inhibitors reaching binding activities in the nanomolar range (Table 6)[112].

Thus, Diederich *et al.* showed that affinity for COMT strongly depends on the size and shape of the linker between the nucleoside and catechol moieties. Adverse effects of hepatotoxicity have been associated with the use of tolcapone, and the hypothesis is that hepatotoxicity might be correlated to the nitrocatechol group[113,114]. Thus, Diederich *et al.* focused their efforts on the development of a new generation of potent bisubstrate inhibitors of COMT lacking the nitro group. Previously, they reported a potent bisubstrate inhibitor (44a, IC_{50} = 9 nM), and by crystal-structure analysis and kinetic study they demonstrated that 44a bound both the SAM and the substrate binding sites of COMT[112]. Therefore, based on this result they modified 44a by replacing the nitro group by a hydrophobic group[115]. This study provided the first family of bisubstrate inhibitors



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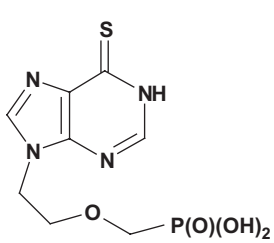
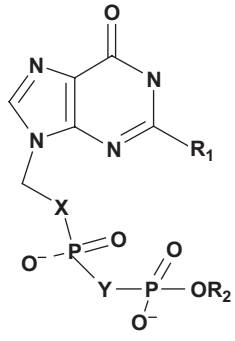
Scheme 23. Multisubstrate adduct for human erythrocyte purine nucleoside phosphorylase (PNPase) (1).



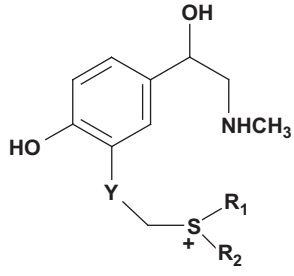
PME-6-Thio-Gua



37

a X = OCH_2CH_2O, Y = O, R_1 = NH_2, R_2 = H
b X = $(CH_2)_n$ = 4, 5, 6, 7, Y = CH_2, R_1 = NH_2, R_2 = H

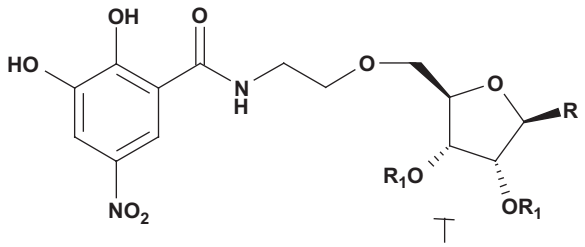
Scheme 24. Multisubstrate adducts for human erythrocyte PNPase (2).

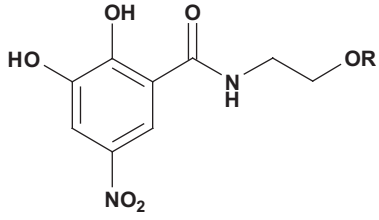


38

R_1 = Ado or $(CH_2)_2CHNH_2CO_2H$
R_2 = Me

Scheme 25. Multisubstrate for catechol *O*-methyltransferase (COMT) (1).

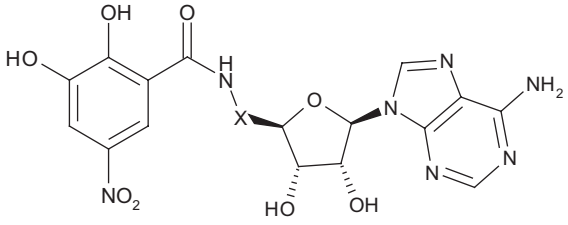




42

Scheme 26. Multisubstrates for COMT (2).

Table 6. IC_{50} values for the bisubstrate inhibitors 43 and 44 of catechol *O*-methyltransferase (COMT).



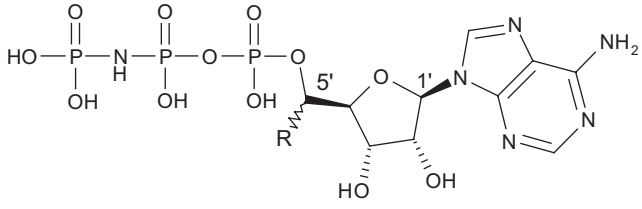
43 44

X = $CH_2CH_2OCH_2$, IC_{50} = 2 μM

a X = CH_2CH=CH, b X = $(CH_2)_{n = 1, 2, 3, 4}$

Compound	*n*	IC_{50} (μM)
43	—	2
44a	—	0.009
44b	1	90
44b	2	0.06
44b	3	0.2
44b	4	5

Table 7. Inhibition constants of adenine nucleotide derivatives on M-2 and M-T.



45 (5′R): R = CH_2-L-$SCH_2CH_2CH(NH_2)CO_2H$

46 a (5′S): R = CH_2CH_2-L-$SCH_2CH_2CH(NH_2)CO_2H$

46 b (5′R)

	K_i for given type of inhibition (μM)			
	M-2		M-T	
Compound	ATP varied	Met varied	ATP varied	Met varied
45	0.13 (C)[a]	0.65 (C)	0.21 (C)	0.67 (M)
46a,b	0.27 (M)	5.4 (C)	0.41 (M)	2.7 (M)
46a or 46b	(0.11)[b]	(2.1)	(0.16)	(1.1)

[a]C, competitive; NC, non-competitive; M, mixed C and NC.

[b]The figures in parentheses are K_i values calculated from the assumption that all the observed inhibitions by 46a,b are mediated solely by the minor component (39% of the total), be that 46a or 46b.

of COMT with an IC_{50} in the nanomolar range. In addition, this demonstrated that the nitro group was not required for a high binding activity[112].

4.6.2. Methionine S-adenosyltransferase isozyme

Methionine *S*-adenosyltransferase (MAT; EC: 2.5.1.6) is an enzyme which catalyzes the attack of the sulfur atom of L-methionine on C5′ of ATP[116–118]. Hampton *et al.* reported the synthesis of new potential inhibitors for two isozymes of MAT: M-2 and M-T[116–118]. A preliminary study looked at the effect of the distance between S and C5′ in the covalent adduct 45 of L-methionine and β,γ-imido-ATP[116–118]. Compounds were tested as inhibitors of M-2 and M-T forms of methionine *S*-adenosyltransferase (Table 7).

In contrast with the previous polyphosphate compounds, the incorporation of a phosphoramidate increased the activity 2.5-fold, with improved metabolic stability. These results showed that compound 45 was the most potent inhibitor for both isozymes M-2 and M-T. The carbon chain was increased in 45–46, which slightly decreased the inhibitory activity. Ribose-P• elongation by one methylene carbon between C4•-C5•, and also between C5•-C6•.

The results showed that this elongation was compatible with the maintenance of dual-site inhibition activity, but did not exhibit more selectivity. It was also found that replacement of the 6-amino group of the ATP-moiety by S-nBu gave a compound three-fold more selective for the target isozyme. Hampton reported a new series of potential inhibitors which were evaluated and compared for potency with 45[116–118,120].These compounds showed an inhibitory activity comparable to 45 but were found to have great selectivity between M-2 and M-T when methionine was used as substrate.

4.6.3. Indole N-methyltransferase

Indole *N*-methyltransferase (INMT) is an *S*-adenosylmethionine (SAM)-dependent enzyme. It catalyzes the conversion of tryptamine into *N*-methyltryptamine (NMT) and *N,N*-dimethyltryptamine (DMT)[121]. Crooks reported the design of a selective *in vivo* inhibitor of tryptamine *N*-methylation which did not disturb other SAM-dependent methylation reactions. This study aimed to determine the role of the tryptamine *N*-methylation in psychotic disorders based on the definition of the COMT transition state. Crooks proposed a similar structure (Scheme 27) for the INMT reaction, with tryptamine as substrate[121]. This structure, 47, represented a stable synthetic analog of this transition state in which the side-chain amino group of tryptamine and the methyl, which migrates, were replaced by a saturated carbon chain.

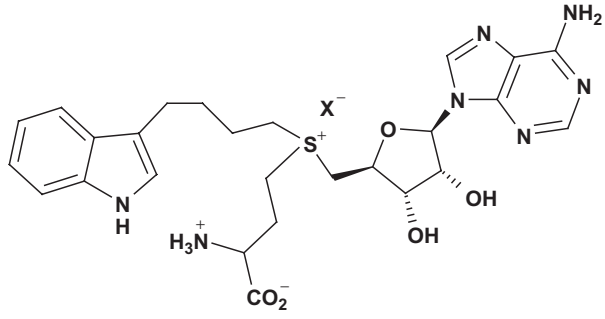
Therefore, the synthesis was directed toward the thioether precursors of 47 and their analogs, methylsulfonium salts (Scheme 28). These compounds were evaluated as inhibitors of INMT, and the most potent inhibitor appeared to be 48a (Y = MeS^+X^-, n = 2, X^- = ClO_4^-, IC_{50} = 38 μM). The lack of inhibitory activity of thioether structures 49b (Y = S, n = 1, 2) and 48b (Y = S, n = 1, 2) suggested that a positive charge borne by the sulfur atom was required for INMT binding.

4.7. *Thymidine phosphorylase*

Thymidine phosphorylase (TPase; EC 2.4.2.4) has been implicated in angiogenesis and chemotaxis in human tumors[122,123]. Over-expression of TPase has also been implicated in inflammatory disease states including rheumatoid arthritis and psoriasis[124,125]. TPase catalyzes the reversible phosphorolysis of thymidine to thymine and 2-deoxyribose 1-phosphate[126]. Balzarini *et al*. reported the first series of MAIs for TPase (Scheme 29) in order to obtain detailed enzymatic kinetics of the bacterial TPase and identification of two different enzyme binding sites[127].

The inhibition kinetics of these compounds against bacterial TPase showed clearly that 52 and 53 inhibited TPase in a competitive or mixed fashion. The K_i values were 3.2, 2.1, 476, and 125 μM for 50, 51, 52, and 53, respectively.

Recently, Allan *et al*. described a novel series of multisubstrate analogs based on compound 55a, synthesized by Li and Gamen[128,129]. These compounds were evaluated as inhibitors of human recombinant TPase (Table 8). Unfortunately, the replacement of the phosphonate group with a carboxylic acid (54b) reduced the inhibitory activity.



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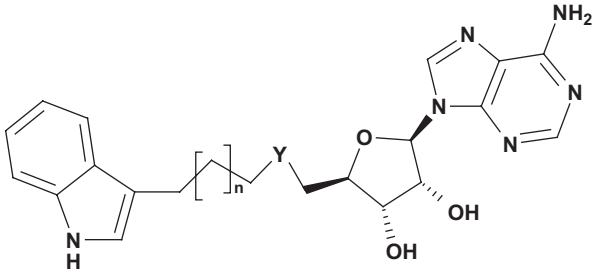
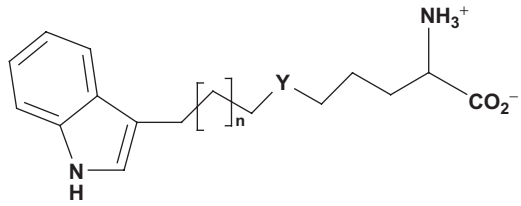
Scheme 27. Synthetic transition-state analog of indole *N*-methyltransferase (INMT).



48

a Y = MeS^+X^-, n = 1, 2 X^- = $CH_3SO_4^-$, ClO_4^-;
b Y = S, n = 1, 2



49

a Y = Me^+X^-, n = 1, 2 X^- = $CH_3SO_4^-$, ClO_4^-
b Y = S, n = 1, 2

Scheme 28. Multisubstrates for INMT.

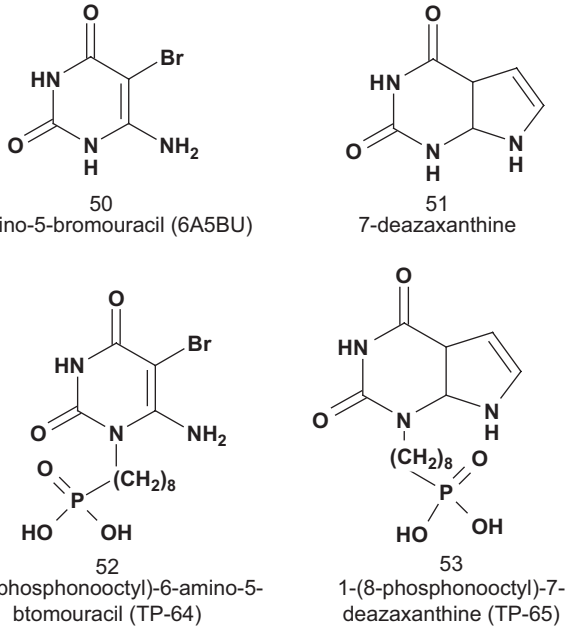
The substitution to 54c rendered the compound inactive. For both types of analog, the endo-isomer was 30–60 times more active than the exo-compound but less active than 54a[128].

4.8. Glycosyltransferases

4.8.1. α-1,2-Fucosyltransferase

α-1,2-Fucosyltransferase is known to transfer a fucosyl residue from guanosine 5′-diphosphofucose (GDP-fucose) to the 1-OH group of β-D-galactopyranosides with inversion of configuration at the fucopranosyl anomeric carbon. Hindsgaul *et al.* reported the first specific glycosyltransferase inhibitors using the mechanism-based approach (Scheme 30) to design MAIs[130].

First, the bisubstrate 57 was kinetically evaluated using the membrane-bound form of fucosyltransferase. The mode of inhibition was competitive with regard to both GDP-fucose (K_m = 7.3 μM) and β-phenyl galactopyranoside (acceptor, K_m = 2600 μM), with K_i values of 16 μM and 2.3 μM, respectively. In contrast, K_i values of GDP-fucose and β-phenyl galactopyranoside were 7.3 μM and 2.6 μM, respectively. These data proved that inhibitor 57 could occupy both the GDP and acceptor binding sites as required for a true bisubstrate analog[130].



Scheme 29. Multisubstrates for thymidine phosphorylase.

Second, further experiments were performed to evaluate the contribution of GDP in inhibitory activity. It was found that 58 (phosphonate) was a competitive inhibitor with respect to the acceptor and a mixed inhibitor with regard to GDP-fucose[130]. It was concluded that the binding properties of 57 were strongly dependent on recognition of the GDP moiety, since the K_i of 58 was increased over 50-fold compared with 57. The bisubstrate analog was also found to be an effective inhibitor of the soluble form of α-1,2-fucosyltransferase.

4.8.2. β-1,4-Galactosyltransferase

Hashimoto *et al.* reported the synthesis of a multisubtrate inhibitor of glycotransferase and more particularly β-1,4-galactosyltransferase (GlcNAc; EC 2.4.1.22)[131]. This multisubtrate was composed of three components: an electrophilic glycosyl residue (Gal), a nucleotide-leaving group (UDP), and a nucleophilic glycosyl acceptor (GlcNAcβ-OMe) (Scheme 31).

This multisubstrate 59 adduct, the design of which was based on a hypothetical mechanism, showed a remarkably potent inhibitory activity toward β-1,4-galactosyltransferase from bovine milk (K_i = 1.35 μM for acceptor GlcNAc and K_i = 3.3 μM for donor UDP-Gal)[131,132]. In addition, this compound was the first multisubstrate example that showed inhibitory activity against GlcNAc.

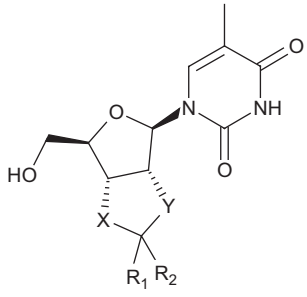
Later, Guillerma *et al.* reported the synthesis of a bisubstrate analog prototype featuring a five-membered ring azasugar with L-xylose stereochemistry, in order to mimic the half chair conformation of the glycosyl cation. A difluoromethylphosphonate group was incorporated at the C1 position to resemble the first position of the pyrophosphate moiety of the donor. They also introduced an amino group in the linker between the azasugar and GlcNAc, expecting an additional electrostatic interaction between the 4-position of the acceptor and the enzyme[132]. This bisubstrate was tested as an inhibitor for chitin synthase (an important target for antifungal agents), but showed no activity.

4.8.3. Sialyltransferase

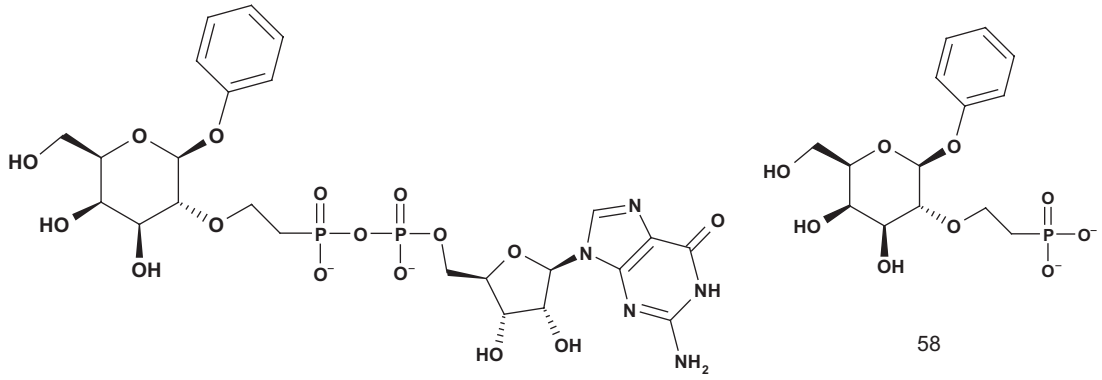
Sialyltransferases (STs; EC 2.4.99.2) are involved in biosynthesis of the sialic acid-containing oligosaccharides[133]. Most

Table 8. Inhibition of thymidine phosphorylase (TPase) by multisubstrate analogs.

Compound	K_i (μM)
54a	0.236 ± 0.007
54b	43.5
54c	No inhibition
55a	8.03 ± 0.18
55b	>500
56a	1.05 ± 0.07
56b	33.4 ± 4.0

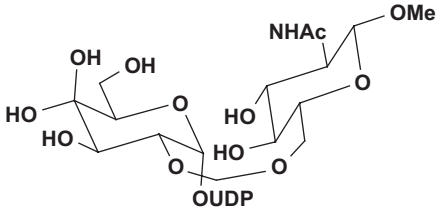


54 X = Y = O; R_1 = H
a R_2 = $CH_2PO_3H_2$;
b R_2 = CH_2COOH;
c R_2 = CH_2CONH_2
55 X = O, Y = CH_2
a R_1 = $CH_2PO_3H_2$, R_2 = H;
b R_1 = H, R_2 = $CH_2PO_3H_2$
56 X = CH_2, Y = O
a R_1 = $CH_2PO_3H_2$, R_2 = H;
b R_1 = H, R_2 = $CH_2PO_3H_2$



57 58

Scheme 30. Multisubstrates for α-1,2-fucosyltransferase.



59

Scheme 31. Multisubstrate for β-1,4-galactosyltransferase.

STs use cytidine-5′-monophospho-*N*-acetylneuraminic acid (CMP-Neu5Ac) as common donor substrate. Several stable donor analogs of glycosyltransferase were synthesized, and Schmidt *et al*. reported the most potent inhibitor with a K_i value of 29 nM[134–141]. The same group also synthesized several multisubstrate adducts, but none had inhibitory effect. Hinou *et al*. synthesized analogs with K_i values similar to the K_m value of CMP-Neu5Ac[142]. Based on a similar idea, Hashimoto *et al.* reported the synthesis of donor analogs 60a–d of CMP-Neu5Ac, the multisubstrate adduct 60e (Scheme 32), and their evaluation as inhibitors of α-2,3-ST and α-2,6-ST (Table 9)[143].

Only the carboxylate derivative showed interesting inhibitory activities against both STs (IC_{50} = 0.047 mM for α-2,3-ST and 0.34 mM for α-2,6-ST). The difference between these two IC_{50} values was about one order of magnitude, so compound 61a was postulated to be a good lead structure for the design of selective bisubstrate analog inhibitors.

4.8.4. α-1,3-Fucosyltransferase

After having developed bisubstrate inhibitors of β-1,4-galactosyltransferase, Hashimoto used the same concept to synthesize bisubstrate analogs targeting α-1,3-fucosyltransferase[144]. Two compounds were designed as bisubstrate analogs for α-1,3-fucosyltransferase, and their inhibitory activities were determined against both α-1,3-fucosyltransferases (α-1,3-FucT) V and VI (Table 10).

These compounds were found to be weak inhibitors for FucT-V but substrates of FucT-VI. This discovery provided new insight into the substrate binding site of these two gene products, and useful information for the development of FucT-V-specific inhibitors. It also helped establish the utility of FucT-VI in order to modify fucosylated glycoconjugates[144].

4.8.5. N-acetylglucosaminyltransferase

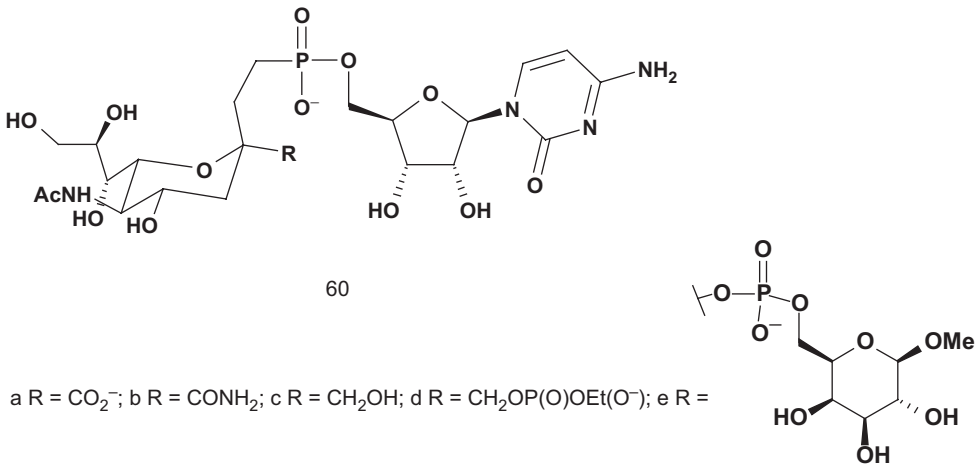
N-Acetylglucosaminyltransferases (GnTs; EC 2.4.2.51) are key enzymes in the production of branched complex *N*-glycan structures. GnTs transfer an *N*-acetylglucosamine (GlcNAc) residue to the core α-1,6-mannose (Man) to form a β-1,6 linkage[145,146]. GnT-V affects T-cell activation and angiogenesis[147–152]. Therefore, inhibition of GnT-V might have potential in the treatment of cancer. Several GnT-V inhibitors were synthesized based on modification of the acceptor substrate oligosaccharide.

Yukishige *et al.* turned their attention to designing multisubstrates relying on the incorporation of a donor into an acceptor, expecting to enhance binding and inhibitory activity[153]. They reported the synthesis of a prototype for bisubstrate-type inhibitors of GnT-V and GnT-IX (Scheme 33). This type of bisubstrate was designed to contain both donor (UDP-GlcNAc) and acceptor components[154]. As the acceptor component, the trisaccharide (GlcNAcb-1,6-Man) was incorporated, as it had previously been reported to serve as an efficient acceptor substrate of GnT-V[155].

This compound 67a was evaluated toward GnT-V and GnT-IX (Table 11). The affinity of 67a to GnT-V was weak compared with the acceptor substrate (K_m = 150 μM). However, the activity toward GnT-IX was more important, so much so that 67a could be used as a probe to investigate the kinetic mechanism of GnT-IX.

The same group developed several analogs of 67a. They studied the dependence of the length of the linker Y (Scheme 33) and evaluated these compounds against GnT-V and GnT-IX (Table 11)[153]. The results showed that inhibition was clearly dependent on the length of the linker. For GnT-V, compounds which had the longest linker showed the strongest activity, with a single exception, 67a.

The correlation between linker length and activity was significantly different between these enzymes. Although these enzymes are homologous, the biological results suggested



Scheme 32. Multisubstrates for sialyltransferases.

Table 9. Inhibitory activities of 61–65 against rat recombinant α-2,3-sialyltransferase (ST) and α-2,6-ST.

	IC_{50} (mM)	
Compound	α-2,3-ST	α-2,6-ST
61a	0.047	0.34
62b	3.3	4.3
63c	4.2	3.2
64d	0.95	2.3
65e	1.3	2.4

that the distances for donor and acceptor binding sites were quite different.

4.9. Acetyltransferases

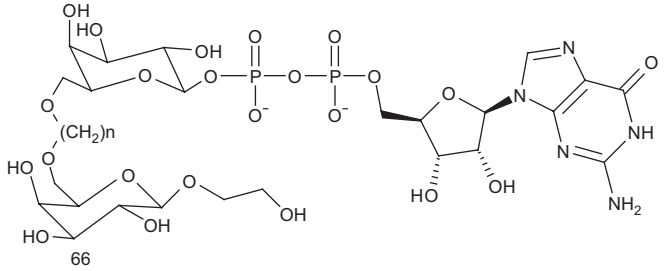
4.9.1. Serotonin N-acetyltransferase

Serotonin *N*-acetyltransferase, also known as arylalkylamine-*N*-acetyltransferase (AANAT; EC 2.3.1.87), catalyzes the transfer of an acetyl group from acetyl coenzyme A (AcCoA) to the primary amine of serotonin to give *N*-acetylserotonin. AANAT catalyzes this transfer via a ternary complex kinetic mechanism; therefore, it is relevant to assume that bisubstrate analog inhibitors composed of tryptamine linked with CoA might result in potent inhibition[156,157]. Bisubstrate analog inhibitors have been used along with AANAT to solve the X-ray structure of the AANAT–bisubstrate complex, which has improved understanding of molecular recognition and the catalytic mechanism.

Cole *et al.* directed their studies toward the design of bisubstrates in which tryptamine was linked to CoA via an acetyl bridge[158]. This compound displayed a K_i value about 1000-fold lower than the substrate K_m value (68a). Many studies developed several bisubstrate analogs with substitution in the indole, CoA, and linker moieties, and these were evaluated as AANAT inhibitors. Studies revealed that the methylene extension of the linker (68b) led to inhibitors as potent as 68a. They also demonstrated that the attachment of an extra methylene group in the linker (68c) led to even more potent inhibition (Scheme 34).

Later, the same group concentrated its attention on preparing a series of novel bisubstrate ketone analogs, deaza

Table 10. Inhibitory activities of bisubstrate analogs 66a and 66b.

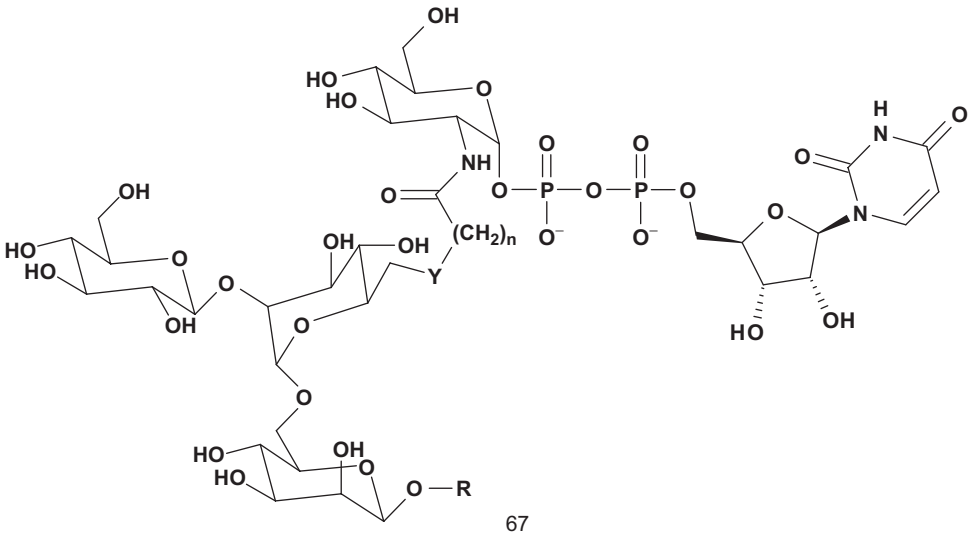


	IC_{50} (mM)		
Compound	FucT-V	FucT-VI	K_i (μM)[a]
66a	0.26	0.11	41
66b	0.27	0.19	43

[a]K_i values for FucT-V.

analogs of 68b, hoping to gain further insight into the role of the linking region in AANAT inhibition[156]. One of these analogs was shown to have an apparent K_i about two-fold less than that of the parent compound 68a[159]. This demonstrated that the amide nitrogen of 68a was not important for a high affinity interaction with AANAT. The study of these bisubstrate ketone analogs revealed that the tetrahedral intermediate bisubstrate mimic only weakly blocked AANAT action.

In addition, the relative orientation of the two substrate moieties with respect to each other appeared to be important for potent inhibition. With the aim of potentially enhancing inhibition and gaining new mechanistic insights, Cole *et al.* reported further modifications of previously synthesized bisubstrates (Scheme 35)[156,160]. These AANAT bisubstrate analog inhibitors allowed for hydrogen binding and electrostatic interaction in molecular recognition sites. Analogs 69a–c were designed to investigate the dependence of the inhibitory potency on the distance between the indole, which was kept intact due to its mimicry of tryptamine, and CoASH moiety in AANAT bisubstrate analogs.



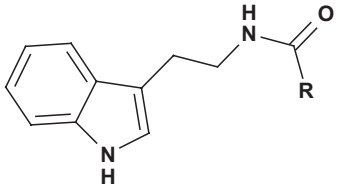
67

a *n* = 1, Y = S

Scheme 33. Bisubstrate for *N*-acetylglucosaminyltransferases GnT-V and GnT-IX.

Table 11. Inhibitory activities of 67 against *N*-acetylglucosaminotransferases GnT-V and GnT-IX.

			GnT-V	GnT-IX
Inhibitor	R	Y	K_i (μM)	
67a	$(CH_2)_8COOMe$	S	71.9	10.1
67b	$(CH_2)_8COOMe$	S-S	119.3	4.7
67c	$(CH_2)_8COOMe$	S-CH_2-S	47.1	17.6
67d	$(CH_2)_8COOMe$	S-$(CH_2)_2$-S	26.9	21.5
67e	$(CH_2)_8COOMe$	S-$(CH_2)_3$-S	18.3	15.1



68

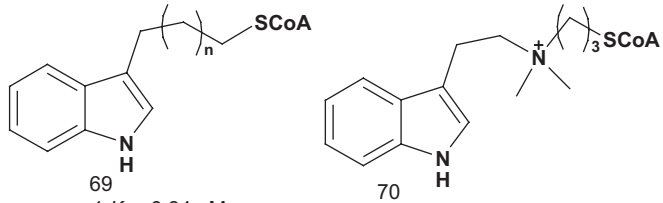
a R = CH_2SCoA (K_i = 48 nM)

b R = CH_2CH_2SCoA (K_i = 67 nM)

c R = $CH(CH_3)CH_2SCoA$ (K_i < 17 nM)

Scheme 34. Bisubstrates for serotonin *N*-acetyltransferase (1).

Bisubstrates 69a–c were evaluated as inhibitors of AANAT (Scheme 35), and these results suggested that the linker's ability to undergo hydrogen-bond interactions made only modest contribution to the enzyme–adduct complex's stability. In addition, the linker between the indole and CoASH moieties required at least four methylenes to allow for strong AANAT inhibition. Compound 70 evaluation showed that the AANAT active site was not adapted to support a positively charged linker.



69

a *n* = 1 K_i = 0.84 μM

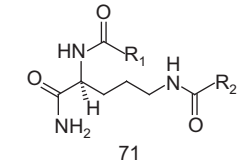
b *n* = 2 K_i = 0.31 μM

c *n* = 3 K_i = 0.36 μM

70

K_i = 5.84 μM

Scheme 35. Bisubstrates for serotonin *N*-acetyltransferase (2).



71

a R_1 = Me, R_2 = $CH(CH_3)SCoA$

b R_1 = Me, R_2 = CH(Ph)SCoA

c R_1 = Me, R_2 = $(CH_2)4SCoA$

d R_1 = Me, R_2 = $(CH_2)7SCoA$

e R_1 = Ph, R_2 = CH_2SCoA

f R_1 = Ph, R_2 = $CH(CH_3)SCoA$

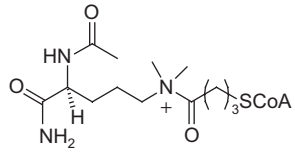
g R_1 = Ph, R_2 = $(CH_2)7SCoA$

h R_1 = Et, R_2 = CH(Ph)SCoA

i R_1 = Et, R_2 = $(CH_2)4SCoA$

j R_1 = $(CH_2)_3CO_2$

Lys-CoA R_1 = Me, R_2 = CH_2SCoA

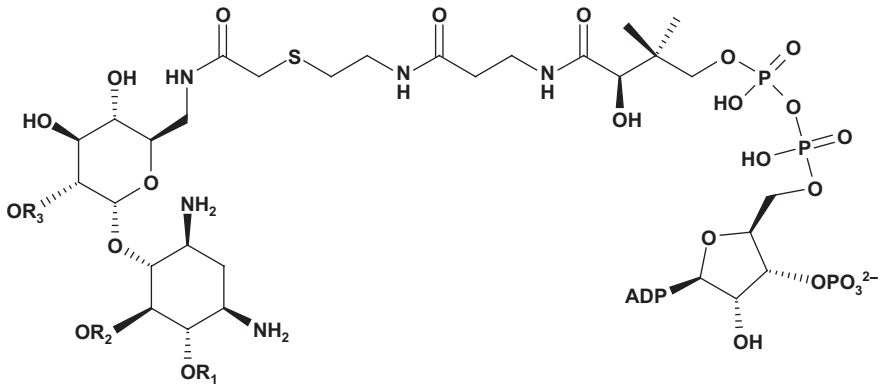


72

Scheme 36. Bisubstrate analogs 71 and 72 for histone acetyltransferases.

4.9.2. Histone acetyltransferases

Histone acetyltransferases (HATs; EC 2.3.1.48) are enzymes which acetylate conserved lysine amino acids on histone proteins by transferring an acetyl group from acetyl CoA to lysine to form ε-*N*-acetyl lysine. Cofactors



a R_1 = H, R_2 = H, R_3 = NH_2, K_i = 76 nM

b R_1 = 3-amino-glucose, R_2 = H, R_3 = OH; K_i = 111 nM

c R_1 = H, R_2 = ribose, R_3 = NH_2, K_i = 119 nM

Scheme 37. Bisubstrate analogs for aminoglycoside 6′-*N*-acetyltransferases (1).

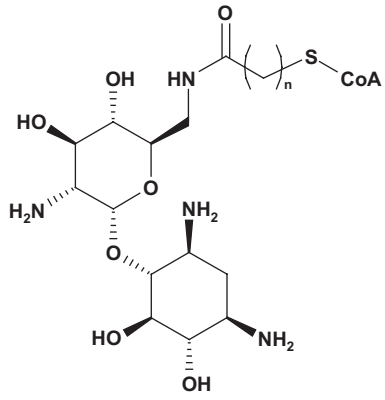
p300 and CBP (CREB binding protein) have been shown to be major regulators of gene expression via their HAT function[161]. In addition, p300/CBP HAT activity appears to be enhanced in some types of cancer and, thus, selective p300/CBP HAT inhibitors may have utility as therapeutic agents[162–164].

Cole *et al.* reported several studies of the structure and substrate processing, before designing bisubstrate analog inhibitors (Scheme 36)[164–168]. These bisubstrates were analogs with Lys-CoA, and studies of the substructures of the CoA moiety of Lys-CoA revealed that the CoA, without modification, was crucial[169].

The Lys-CoA analogs were tested against the catalytic domain of p300 and they were referenced to Lys-CoA. Most compounds showed IC_{50} values similar to or worse than those of Lys-CoA. Modification of the linker with phenyl substitution or homologation of the alkyl chain reduced the inhibitory activities. In addition, deletion of the carbonyl group from the linker decreased the inhibitory potency. Compared to Lys-CoA, only 71a and 71e enhanced the potency with IC_{50} values of 0.8 μM and 0.7 μM respectively, about four-fold lower than Lys-CoA. In contrast, the double modification operated with 71f showed a similarly potent inhibition to that of Lys-CoA, indicating a cancelation of the affinity-enhancing effects of each substitution.

4.9.3. Aminoglycoside 6′-N-acetyltransferase (AAC(6′)-li)

Aminoglycosides are efficient antibiotics and are particularly active against aerobic gram-negative bacteria[170]. Nevertheless, the emergence of aminoglycoside resistance has strongly restricted their use as antibacterial agents[171]. Auclair *et al.* developed a regio- and chemoselective methodology for direct *N*-6′-derivatization of unprotected aminoglucosides, facilitating the preparation of a first series of AAC(6′)-li bisubstrate analog inhibitors (Scheme 37)[172,173].



74

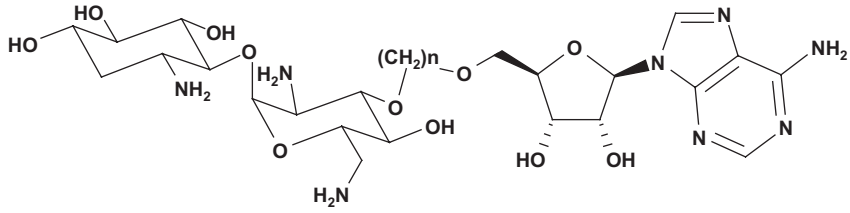
a *n*=2; b *n*= 3; c *n*=4

Scheme 38. Bisubstrate analogs for aminoglycoside 6′-*N*-acetyltransferases (2).

These target bisubstrates were designed according to the proposed tetrahedral intermediate that resulted from the attack of aminoglycoside 6′-NH_2 on the thioester carbonyl of AcCoA in the active site of the enzyme[174]. Kinetic studies of 73a–c showed these bisubstrates as potent competitive inhibitors, with nanomolar K_i values. Based on these results, Auclair synthesized a novel series of bisubstrates (Scheme 38) to investigate the importance of the size and geometry of the linker[156–160,173].

AAC(6′)-li inhibition assays showed that 74a was the most potent bisubstrate inhibitor of this series. The enzyme bound 74a about 200-fold tighter than its natural substrate AcCoA (K_m = 9.6 μM). It was also observed that an increase in length of the linker led rapidly to a decrease in activity.

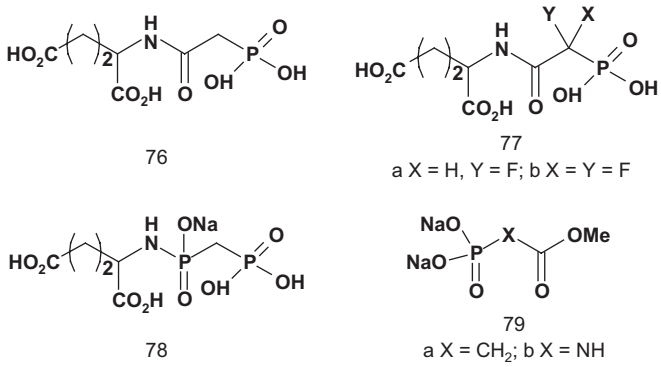
The high potency of these bisubstrate inhibitors allowed crystallization of AAC(6′)-li with aminoglycoside derivatives that provided valuable guidance in further studies of



75

a n = 5; b n = 6; c n = 7; d n = 8

Scheme 39. Bisubstrate analogs for aminoglycoside 3′-phosphotransferases.



76

77

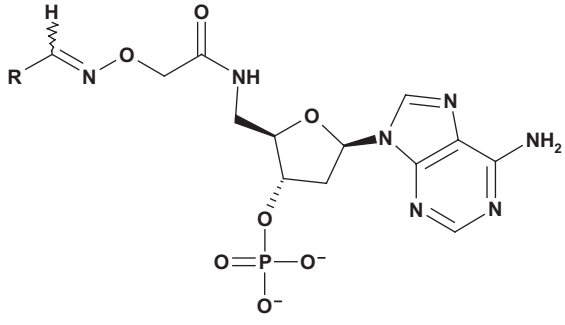
a X = H, Y = F; b X = Y = F

78

79

a X = CH_2; b X = NH

Scheme 40. Bisubstrates, transition-state analog, and competitive inhibitors for aspartate transcarbamylase (ATCase).



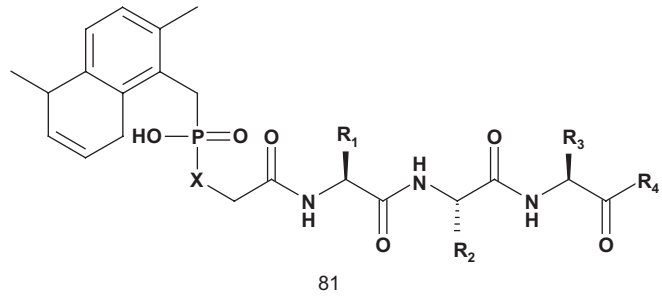
80

R = alkyl, aryl, polyaryl

Scheme 41. Library of bisubstrate analogs for sulfotransferases.

this enzyme. Recently, the same group reported a novel series of bisubstrate inhibitors of AAC(6′)-li in order to carry out a structure–activity relationship study[175]. The inhibitory activity of this new generation of bisubstrate analogs was evaluated with AAC(6′)-li, but none had a low, nanomolar K_i value. The SAR from these studies indicated that inhibition by aminoglycoside-CoA bisubstrates was more sensitive to truncation at the aminoglycoside than at the CoA end.

Targeting the same family of enzymes, Mobashery *et al.* reported the development of tethered bisubstrates (Scheme 39)[176]. They focused their attention on aminoglycoside 3′-phosphotransferases (APH3′; EC 2.7.1.95), which catalyze the transfer of the γ-phosphoryl group of ATP to the 3′-hydroxyl of aminoglycosides. APH3′ enzymes were



81

Scheme 42. Bisubstrate analogs for farnesyl transferase (FPT) (1).

the cause of the demise of kanamycin treatment in clinical trials.

Compounds 75a–d were tested against APH3′ Ia and IIa as potential inhibitors. All of them were competitive inhibitors for the enzyme, and 75 showed the best inhibitory activity with a K_i value of 3 μM and 9 μM, respectively, when the substrate of the enzyme was kanamycin A (K_m = 4 μM).

4.9.4. Aspartate transcarbamylase

Aspartate transcarbamylase (ATCase; EC 2.1.3.2) catalyzes the first unique step of the pyrimidine pathway, which is carbamylation of the amino group of L-aspartate by carbamylphosphate to produce *N*-carbamyl-L-aspartate[177]. This enzyme is an interesting target for the development of antiproliferative drugs, and numerous approaches to the design of new antitumoral agents were based on the search for ATCase inhibitors[178,179]. So far, the most potent synthesized inhibitor is the bisubstrate analog, *N*-(phosphonoacetyl)-L-aspartate (PALA)[180,181]. PALA is a competitive inhibitor in respect to carbamyl phosphate, but not with L-aspartate.

The application of this bisubstrate has facilitated studies about the regulation, binding characteristics, and crystallographic structure of ATCase[182–186]. More recently, Grison *et al.* investigated the rational design of ATCase inhibitors based on the understanding of the catalytic reaction mechanism[187]. They synthesized several bisubstrate analogs, one transition-state analog, and competitive reaction inhibitors (Scheme 40).

The influence of these compounds was examined on ATCase activity from *E. coli*. Compounds 76, 78, and 77a did not show any inhibitory activity, although, at a concentration of 5 mM, 77b inhibited ATCase at about 45%. Only 79a and 79b showed interesting inhibitory activities. At a

Table 12. Inhibitory activities of 81 against GnT-V and GnT-IX.

Compound	X	R_1	R_2	R_3	R_4	I_{50} (FPT)	I_{50} (GGT-I)
81a	O	-$CHMe_2$	-CH_2CHMe_2	-CH_2OH	OH	60 ± 30 nM	59 ± 10 μM
81b	O	-$CHMe_2$	-$CHMe_2$	-$(CH_2)_2SMe$	OH	6.2 ± 0.5 nM	10 ± 1.7 μM
81c	CH_2	-$CHMe_2$	-$CHMe_2$	-$(CH_2)_2SMe$	OH	6 ± 1 nM	21 ± 9.9 μM
81d		-$CHMe_2$	-$CHMe_2$	-$(CH_2)_2SMe$	OMe	[a]	[a]
81e	CH_2	-$CHMe_2$	-$CHMe_2$	-$(CH_2)_2SMe$	OMe	[a]	[a]
81f	CH_2	-$CHMe_2$	-$CHMe_2$	-$(CH_2)_2SMe$	OPOM	[a]	[a]

[a]Compounds 81d–81f were prepared and evaluated as prodrugs.

concentration of 0.05 mM, the inhibition was about 50%, and rose to 80% at 0.33 mM.

4.9.5. Sulfotransferases

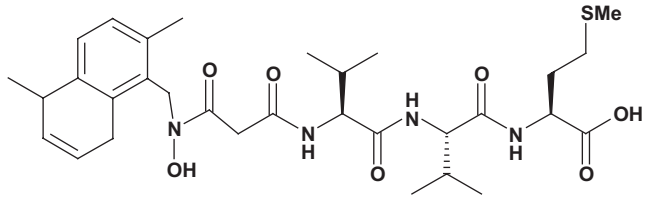
Sulfotransferases (EC 2.8.2.2 [OH] or EC 2.8.2.3 [NH_2]) catalyze the transfer of a sulfuryl group from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to a hydroxyl (or amino) group on a carbohydrate, protein, or small molecule acceptor[188–190]. It has been demonstrated that sulfotransferases have strong connections with several disease states; thus, this discovery has positioned sulfotransferases as important therapeutic targets[191].

Bertozzi *et al*. designed a library of bisubstrate analog inhibitors. Their strategy was to create a library (Scheme 41) possessing two elements: a PAPS mimic for recognition toward sulfotransferases, and a variable hydrophobic and drug-like component to bind the acceptor pocket[192].

All compounds were tested as inhibitors of estrogen sulfotransferase (EST; EC 2.8.2.4), and they displayed greater than 50% inhibition at 200 μM; they also shared a greater than 55% ion abundance decrease in IEMSP (immobilized enzyme mass spectroscometry)[192]. Other bisubstrate adducts, stemming from this study, were further developed (Scheme 41; R = estrone)[193]. These compounds were also tested against EST, and were found to display moderately potent inhibitory activity. Competitive behavior was observed against PAPS (K_m = 2.5 nM) with a $K_{i(comp)}$ of 2.9 nM, and non-competitive behavior against substrate estrone (K_m = 50 nM) with a $K_{i(non\text{-}comp)}$ of 4.0 nM[193].

4.10. Farnesyl/geranylgeranyl transferases

Farnesyl transferase (FPT; EC 2.5.1.58) catalyzes the transfer of a farnesyl residue from farnesylpyrophosphate (FPP) to the thiol of a cysteine side chain of proteins bearing the CAAX-tetrapeptide sequence[194,195]. FPT catalyzes a bisubstrate reaction, and this offered many opportunities for inhibitor design. Thus, several inhibitors were prepared based on either of the two substrates, and examples of both CAAX- and FPP-based inhibitors were reported in the literature[196–200]. Patel *et al.* described the first series of potent bisubstrate analog inhibitors of FPT (Scheme 42)[201,202]. Their strategy in inhibitor design was to keep the farnesyl group of FPP in order to preserve putative hydrophobic interactions, and the C-terminal tripeptide was chosen as the peptide substrate component[201,202]. These two parts were linked via a phosphonic or phosphinic acid linker to replace the sulfhydryl group.



82

Scheme 43. Bisubstrate analogs for FPT (2).

These inhibitors were evaluated for selectivity using the closely related enzyme geranylgeranyl protein transferase type I (GGT-I) (Table 12).

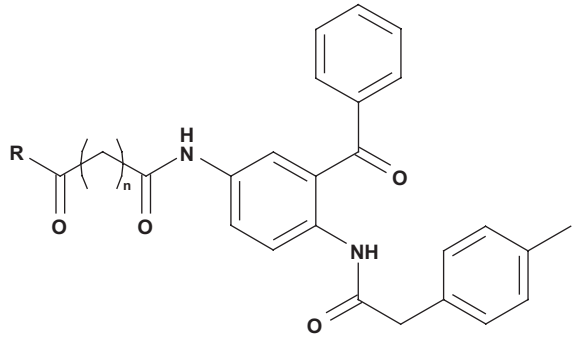
Compounds 81a–f were only moderately active against GGT-I (Table 12), thereby affording greater than 1000-fold selectivity in favor of the targeted enzyme FPT. In contrast, methyl prodrugs 81d and 81e showed a 75–80% decrease in transformed foci at 100 μM concentration. At 100 μM concentration both 81e and 81f almost completely suppressed colony formation of *H*-ras transformed NIH 3T3 cells in soft agar[201,202]. Unlike the tetrapeptide-based inhibitors, a free sulfhydryl group is not a requirement for the activity of these phosphonic and phosphinic acid bisubstrate inhibitors.

Patel *et al.* then developed a series of novel hydroxamic acid-based bisubstrate analog putative inhibitors of FPT. Thus, the farnesyl group and the tripeptide group of the C-terminal CAAX motif were linked together via a hydroxamic acid (R-N(OH)C(O)-R′). The introduction of a hydroxamate group as a linker instead of a branched functional group was to avoid any problems of chirality in this part of the molecule. The replacement of the sulfhydryl group found in tetrapeptide CVLS (I_{50} = 1 μM) by an *N*-methylhydroxamic acid was unsuccessful[203].

Only compound 82 (Scheme 43) was found to be a two orders of magnitude better inhibitor than the first lead, 81. Compound 82 was effective in blocking prenylation of protein in the whole cell, including $p21^{ras}$.

The presence of a free thiol in these types of molecules was a source of adverse drug effects, as observed with the angiotensin converting enzyme inhibitor captopril. Patel *et al* demonstrated that the free sulfhydryl group was not necessary for inhibitory activity[201,202,204]. Hence, Schlitzer *et al.* reported a series of non-thiol farnesyltransferase inhibitors as bisubstrate inhibitors (Scheme 44)[205].

The evaluation of these bisubstrates (83a–e and 84a–b) showed very weak inhibitory activity of the



83 a R = OMe, *n* = 1

b R = OH, *n* = 2

c R = OMe, *n* = 2

d R = OH, *n* = 3

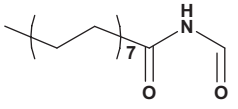
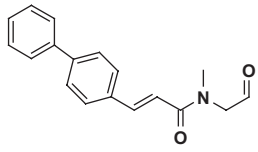
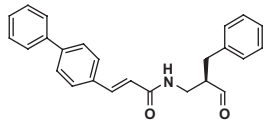
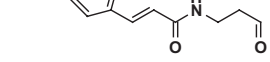
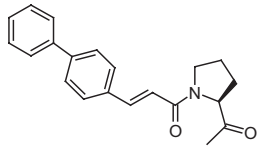
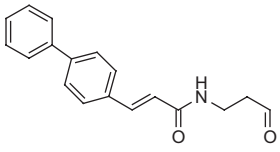
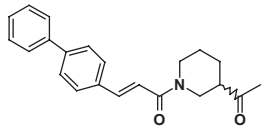
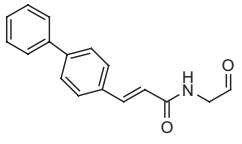
e R = OMe, *n* = 3

84 a R = $NH(CH_2)_{14}$, *n* = 2

b R = $NH(CH_2)_{13}$, *n* = 3

Scheme 44. Bisubstrate analogs for FPT (3).

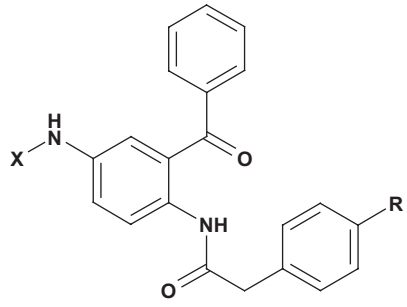
Table 13. Farnesyltransferase inhibitory activity of compounds 85a–i.

Compound	X	R	IC_{50} (μM)	Compound	X	R	IC_{50} (μM)
85a[209]		H	0.4	85f		CH_3	10
85b		H	22	85g		CH_3	32
85c		CH_3	14	85h		CH_3	25
85d		CH_3	9.3	85i		CH_3	30
85e		CH_3	36				

farnesyltransferase, and there was no difference between a carboxylic group terminal and the corresponding methyl ester. This can be explained by the loss of binding energy upon replacement of the thiol by a carboxyl group[205]. Efforts then focused on development of both the peptidic and prenylic substrates and studying the structural requirements of the central moiety[206,207].

Aromatic acrylic acids were then identified as farnesyl mimetics, and Schlitzer *et al.* developed a new series of bisubstrates where the aliphatic farnesyl surrogate was replaced by an aromatic acrylic acid derivative (Scheme 45)[208,209].

Compared to the benzophenone-based bisubstrate analog farnesyltransferase inhibitors (85a)[210], compounds 85b–i, bearing an aromatic farnesyl mimetic instead of an aliphatic moiety substituent, exhibited weak inhibitory



85

Scheme 45. Bisubstrate analogs for FPT (4).

activity against farnesyltransferase (Table 13). However, some of these compounds displayed concentration-dependent cytotoxicity or (at higher concentration) even a cytocidal effect against MCF-7 breast cancer cells. The compounds 85d and 85f were more active than the antitumor drug cisplatin[209].

Geranylgeranyl transferase-1 (GGT-1; EC 2.5.1.59) performs the same reaction as FPT but with geranylgeranylphosphate as a substrate[211]. Substrate specificity toward FPT and GGT-1 is determined by the C-terminal residue X in the CAAX-box motif of Ras or other closely related small GTPases[212]. FPT preferably uses substrates where X is Met, Ser, Gln, or Ala, while GGT-1 favors substrates when X is Leu or Phe[213,214].

Overhand *et al.* developed the next generation of bisubstrate potential inhibitors of GGT-1[215]. They had reported a series of CAAL analogs featuring sugar amino-acid based dipeptide isosteres as replacement of the central AA dipeptide, and the most potent compound (Scheme 46) had an IC_{50} value of 68 μM[216].

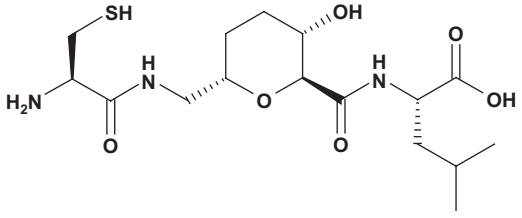
To enhance the potency, Overhand *et al.* attached isoprenyl moities to these CAAL analogs, leading to a bisubstrate mimic[215]. This generation of bisubstrate analogs was evaluated as inhibitors of GGT-1, and two compounds revealed interesting inhibitory properties (Scheme 47). Compounds 87a ($IC_{50} = 12.7 \pm 1.3\,\mu M$) and 87b ($IC_{50} = 12.3 \pm 1.0\,\mu M$) inhibited GGT-1 with equal efficiency, although they differed in the nature of the side chain.

4.11. Riboflavin synthase

Riboflavin synthase (EC 2.5.1.9) catalyzes an unusual dismutation reaction involving the transfer of a four-carbon unit from one molecule of 6,7-dimethyl-8-D-ribityllumazine bound at the donor site of the enzyme to a second identical lumazine molecule located at the acceptor site of the enzyme. This transfer results in the formation of one molecule of riboflavin and one of pyrimidinedione[217–219]. Due to the fact that riboflavin synthase uses two identical molecules of lumazine, the MAI approach was used by Cushman *et al.* to identify potential riboflavin synthase inhibitors. They developed a series of potential bisubstrate adducts by attaching two units of 6,7-dimethyl-8-D-ribityllumazine with a carbon chain as linker. Additionally, they also examined the activity of the two units of pyrimidinedione coupled together with the same linker (Scheme 48)[220].

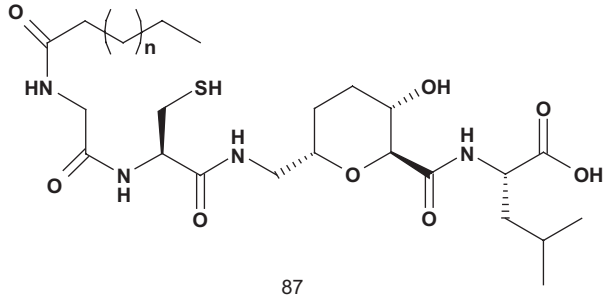
Only the bis(nitro-uracils) 88a–b and bis(luminazines) 89a–c were evaluated against riboflavin synthase and lumazine synthase.

The most potent inhibitor proved to be 89b, with a K_i value of 37 μM and a C-3 carbon chain linker. This greater relative potency suggested that 89b might act as a bisubstrate adduct inhibitor[220].



86

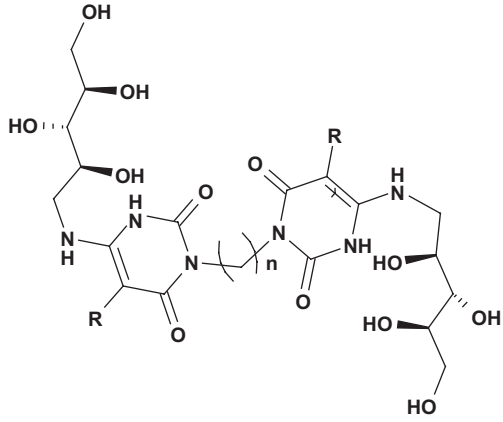
Scheme 46. Bisubstrate analogs for geranylgeranyl transferase-1 (GGT-1) (1).



87

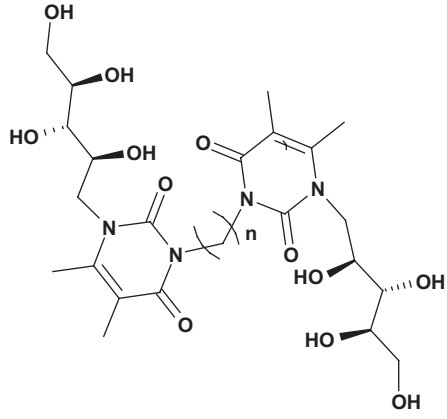
a *n* = 12; b *n* = 14

Scheme 47. Bisubstrate analogs for GGT-1 (2).



88

a R = NO_2, *n* = 3, 4, 5; b R = NH_3Cl, *n* = 3, 4, 5



89

a *n*= 3; b *n* = 4; c *n* = 5

Scheme 48. Bisubstrate adducts for riboflavin synthase.

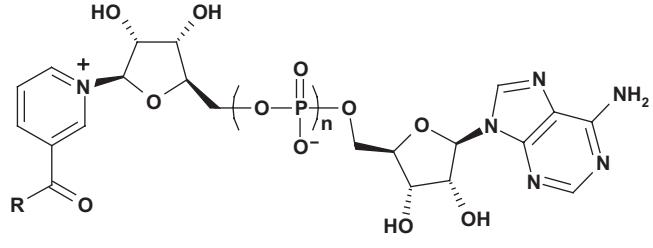
4.12. Nicotinamide mononucleotide adenyltransferase

Nicotinamide mononucleotide transferase (NMNAT; EC 2.7.7.1) catalyzes the synthesis of NAD^+ from nicotinamide mononucleotide (NMN) and adenosine triphosphate (ATP). It is the last step in both *de novo* and salvage NAD^+ biosynthetic pathways, and is an essential protein in all organisms[221]. NMNAT activity has therefore been identified as a potential target for development of novel chemotherapies, and Magni *et al.* developed several multisubstrate adducts (Scheme 49) as putative inhibitors of NMNAT[222].

The NMNAT catalytic reaction proceeds via a ternary complex formation; therefore, the enzyme should be inhibited efficiently and specifically by multisubstrate analogs composed with NMN and AMP but connected with one more phosphoryl unit as linker[223]. The evaluation of these multisubstrates did not show great inhibitory activity, but has proved useful for mechanistic and metabolic studies of this enzyme in nicotinamide adenine dinucleotide (NAD) biosynthesis[222].

Conclusion

Over the last four decades, a number of methodologies have been developed and applied in the design of enzyme inhibitors. One such method, the MAI approach, has been used extensively and successfully in the design of inhibitors, which has allowed insights into enzymatic mechanisms and the development of drug leads. The synergistic effect of the covalent association between substrate and cofactor analogs or between multiple substrate analogs allows the creation of inhibitor templates possessing K_i values within the substrate K_m range whilst also achieving satisfactory enzyme selectivity. For many enzymes for which the MAI approach has been adopted, selectivity and affinity have been greatly improved by modification of the building blocks and the linker. Furthermore, the MAI approach has permitted the synthesis of chemical entities to facilitate enzyme co-crystallization studies, providing essential structural knowledge for future inhibitor design. For instance, many research groups have successfully exploited such structural findings to identify secondary binding sites and more accurately determine the distance between substrate and cofactor binding sites to allow more specific design of linker lengths and flexibilities[224–226].



90

a R = NH_2, *n* = 3, 4; b R = OH, *n* = 4

Scheme 49. Multisubstrates for nicotinamide mononucleotide transferase.

In conclusion, the MAI approach provides a reliable method for designing inhibitors, and is most effective when combined with other drug-design tools such as crystallography or modeling. This method shows itself to be extremely useful for the specific probing of biological drug targets, and thus facilitates the early steps of the associated drug discovery programs. Nevertheless, for identification of lead compounds, the MAI approach is far from being appropriate, as most compounds so far reported do not possess drug-like qualities, which are so important to successful drug discovery programs. However, the MAI approach must still be considered as a relevant tool for the development of pharmacophore building blocks, which will facilitate drug design.

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