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Inhibitors of an AdoMet-Dependent 3-Amino-3-Carboxypropyl Transferase and their Use as Ligands for Protein Affinity Chromatography

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Abstract: The screening of potential affinity ligands and the development of a two-stage affinity purification of an *S*-adenosylmethionine-dependent 3-amino-3-carboxypropyl transferase involved in the biosynthesis of the β-lactam antibiotic nocardicin A is described from a cell-free extract of *Nocardia uniformis* subsp. *tsuyamanesis*. This protein is particularly sensitive to ion exchange and salt effects necessitating a pair of neutral ligands to be used in the final purification.

Keywords: β-lactam antibiotics, biosynthesis, protein purification, affinity chromotography

Introduction

Research on the biosynthesis of the β-lactam antibiotics, like other groups of natural products, has advanced in the last decade through isolation of the genomic DNA segments (gene clusters) that ultimately direct their biosynthesis. In bacteria the genes that encode penicillin (**1**), cephalosporin (**2**) and cephamycin (**3**) were the first among the β-lactams to be isolated, and they are grouped together in a large cluster *e.g.* in streptomyces.[1] Next, the genes responsible for clavulanic acid (**4**) biosynthesis were determined.[2-4] Most recently a gene cluster in *Erwinia* has been correlated to the biosynthesis of carbapenem-3-carboxylic acid (**5**), the simplest of over 40 carbapenem antibiotics.[5] The monocyclic β-lactams exemplified by nocardicin A (**6**) constitute the fourth structural family in this important class of natural products. Its biosynthetic genes are, however, unknown. To provide a reliable experimental route to this genomic information, we have isolated a biosynthetic enzyme that is both unambiguously specific to nocardicin biosynthesis, and catalyzes an unusual transformation in its own right.

This paper is dedicated to Professor A. Ian Scott in celebration of his 70th birthday.

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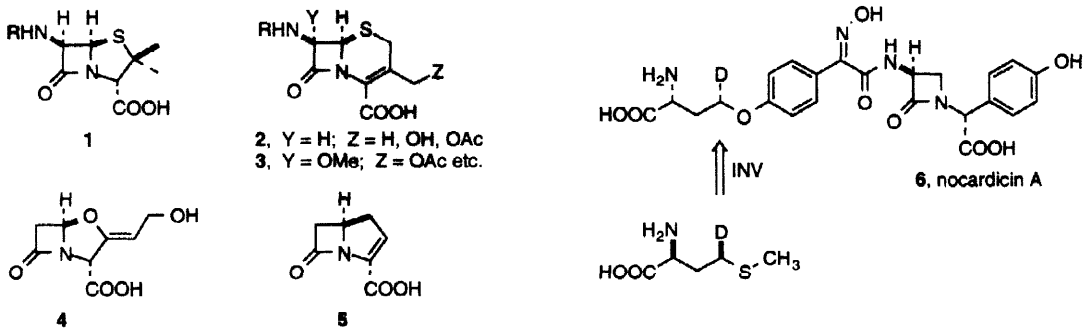
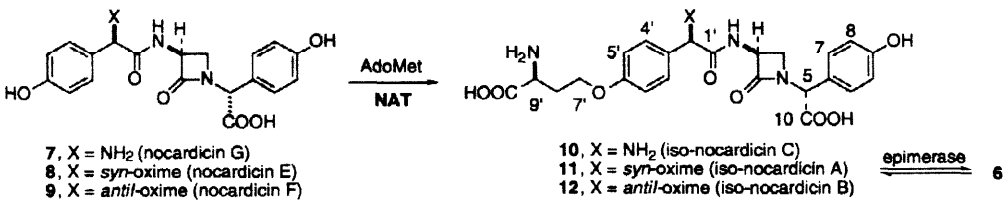
Scheme 1



It is possible through "reverse genetics" techniques to proceed from a purified protein to identify, clone, sequence and over-express its encoding gene. It is generally the case in bacteria that one can also locate in the flanking DNA other biosynthetic genes that characterize a cluster. We have previously shown that a cell-free extract of *Nocardia uniformis* subsp. *tsuyamanesis* (ATCC 21806) can carry out a 3-amino-3-carboxypropyl transfer reaction ("transferase" or NAT) to nocardicins G (**7**), E (**8**) and F (**9**) utilizing *S*-adenosylmethionine (AdoMet) (Scheme 2).[6] This is a rare biochemical fate for AdoMet, a cofactor much more commonly associated with methyl transfers or 3-aminopropyl transfers in polyamine biosynthesis from decarboxyAdoMet. These latter two reactions are known to occur with stereochemical inversion consistent with an S_N2 mechanism and presumed in-line geometry of reaction at the sulfonium center.[7-12] In accord with the observation that AdoMet was similarly required in the nocardicin cell-free reaction, chirally-labeled methionine was found to incorporate into nocardicin A with clean inversion (Scheme 1).[13]

Scheme 2



When purification of NAT was attempted, however, severe problems with the stability of the enzyme were encountered. NAT required 20% glycerol to remain active and was inactivated by ion exchange chromatography, salt gradients, dilution below *ca.* 0.1 mg/mL, and pH below 7 or above 9. The high concentration of glycerol required to maintain activity limited resolution on gel filtration and hydrophobic interaction chromatography. Various dye-ligand chromatographies were also unsuccessful. Thus, a mild, efficient procedure tolerant of high glycerol concentrations became essential to resolve NAT from other cellular proteins.

Affinity chromatography is appealing to organic chemists as a means to selectively purify a protein using a matrix bearing ligands structurally similar to reactants, reaction intermediates or products of the enzyme of interest. Biospecificity is thought to be conferred by the binding discrimination of the active site or an allosteric site. Therefore, substrate analogues, inhibitors and transition state mimics have been used, and often lend the practical advantage of stabilizing the enzyme during isolation. Furthermore, if conditions are chosen carefully, the protein can be gently eluted with an excess of free ligand. On the other hand, difficulties can be encountered in ligand design that are not always easy to overcome. For example, the functional group used to attach the small molecule to the gel might be required for useful binding to the desired protein. The

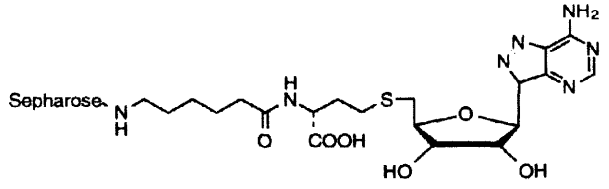
concentration of immobilized ligand should be controlled; if it is too high, non-specific interactions can destroy the resolving power of the column. The length and nature of the spacer arm between the solid support and the ligand may have to be adjusted to allow the macromolecule to access the ligand, but especially with hydrophobic arms, non-specific interactions with the spacer arm can be problematic. Lastly, if the dissociation constant of the protein-ligand complex is small, the protein can bind too tightly, causing problems with elution.[14]

In this paper we describe the development of a highly efficient purification of NAT using two affinity purification steps. By taking advantage of the two substrate-two product reaction scheme, two complementary, biospecific affinity columns were designed that afforded homogeneous protein rapidly and in good yield from a cell-free extract of *N. uniformis*. The ultimate success of this method for the purification of a highly sensitive enzyme, however, was dependent on a stepwise evaluation of potential ligands and the realization of the negative impact ion exchange effects can have from charged affinity ligands and residual reactive sites on the resin itself. The instructive aspects of these experiments are described.

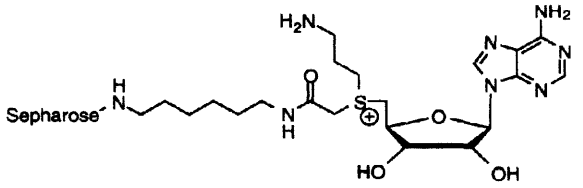
Results

Many enzymes requiring adenosyl sulfur compounds [AdoMet, decarboxylated AdoMet (dc AdoMet), *S*-adenosylhomocysteine (AdoHcy), and 5'-methylthioadenosine (MTA)] as substrates have been purified by affinity chromatography using substrate, product, or an analog of either as ligands.[15-31] For example, AdoHcy/MTA nucleosidase was purified 25-fold using the substrate analog *S*-formycinylhomocysteine-Sepharose.[20] (**13**, Scheme 3) Spermidine synthase was purified 570-fold from bovine brain utilizing *S*-adenosyl-(5')-3-thiopropylamine-Sepharose (decarboxylated AdoHcy, **14**, a substrate analog) as the adsorbent. In this case, the ligand forms a sulfonium linkage to the solid support, closely mimicking the charge distribution of the substrate, decarboxylated AdoMet.[26] The same coupling strategy was employed in the synthesis of AdoHcy-Sepharose, a gel which yielded a 360-fold purification of AdoMet:protein carboxy O-methyl transferase.[15] In solution, AdoHcy is an inhibitor of this enzyme, but once it is attached to the gel, the sulfonium ion produced by the coupling chemistry resembles the substrate AdoMet.

Scheme 3



13, *S*-formicinylhomocysteine-Sepharose

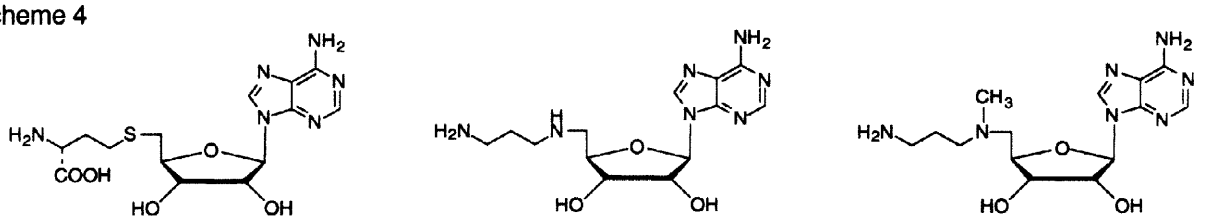


14, decarboxylated AdoHcy-Sepharose

Ligands considered for affinity purification of NAT were first screened by testing for inhibition of the normal reaction in a crude cell-free extract purified only through ammonium sulfate precipitation and dialysis.[6] If inhibition was observed, binding of the potential ligand to the enzyme was presumed and an IC_{50} was determined to assess approximate affinity for the protein. The initial proposals for ligands were analogs of AdoMet, chosen owing to the available precedents and the necessity for the transferase to bind AdoMet. Also, in analogy to the three examples above, a positive charge at the 5' position (sulfonium in AdoMet) was considered to be a necessary component of the ligand. While it was assumed that one or more of these compounds would be an inhibitor of the transferase and, therefore, be a candidate for immobilization, it was

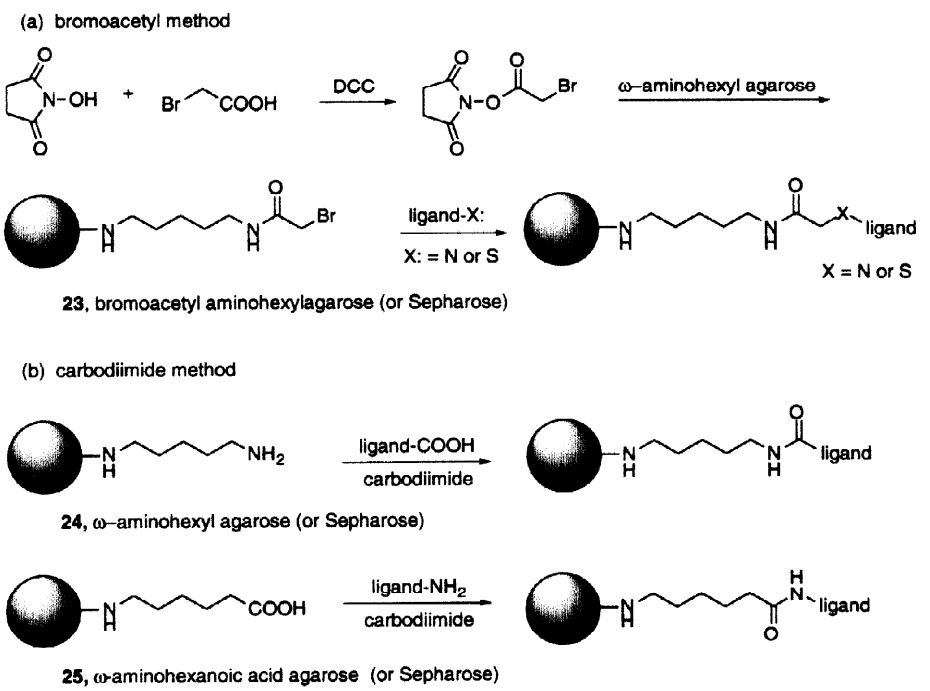
also realized that AdoMet analogs would probably bind other cellular and AdoMet-utilizing proteins in addition to the 3-amino-3-carboxypropyl transferase. This could lead to a lack of specificity and problems with subsequent purification steps if the group of proteins selected was structurally similar.

Scheme 4



15, AdoHcy **16** **17**

AdoHcy (**15**, Scheme 4) was found to be an inhibitor of the transferase, with an IC_{50} of about 1 mM. Therefore, AdoHcy was coupled to Sepharose 4B using the bromoacetyl method (Fig. 1) described by Kim,[15] affording a gel that could have mixed functionality: a sulfonium linkage, precedented by Samejima and Yamanoha,[27,28] or a linkage through the amine as described by Kim.[15] Control of the coupling chemistry should be a function of pH, with the alkaline pH utilized favoring the amino linkage, but control experiments with the gel showed a mixture resulted. This multiplicity was not initially considered detrimental; the mixed coupling of AdoHcy afforded the transferase a steric selection of binding sites, at least one of which was likely to effect efficient adsorption. Unfortunately, the column was found to be chemically unstable and caused irreversible adsorption of NAT activity after the first few runs. Samejima and Yamanoha[27,28] observed a 50% decline in the column capacity of decarboxylated AdoHcy-Sepharose (sulfonium linkage), even when it was stored in 5% acetic acid, which discourages nucleophilic displacement of the thioether leaving group by the solvent.



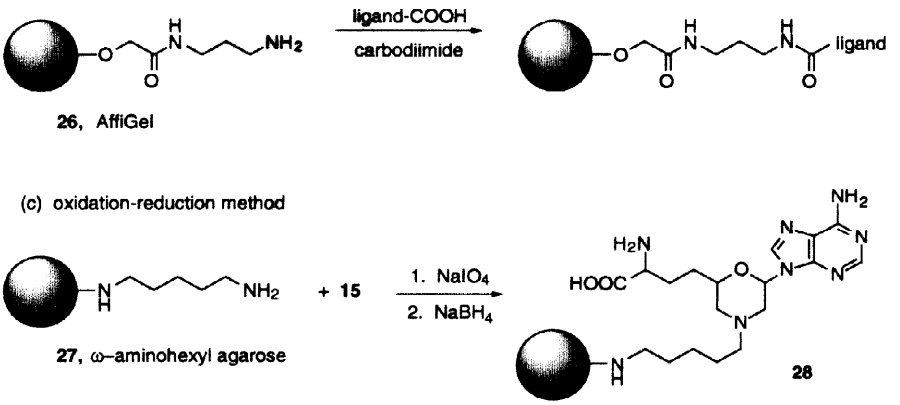


Figure 1. General methods of affinity ligand attachment to solid supports.

Chemically stable ligands that could be attached by an unambiguous synthetic procedure to the solid support were required. The charge at the 5' position was incorporated into the ligand by substituting a nitrogen for the sulfur. This change also improved the stability of the compounds and facilitated their synthesis. From the 5' amino group, an aminopropyl chain was chosen to sterically mimic AdoMet and provide the attachment site to the gel. Thus, aminopropylamino adenosines **16** and **17** (Scheme 4) were synthesized according to Kolb, et al.[32] An IC_{50} of about 1 mM was determined for **16**, while **17** had an IC_{50} of 0.4 mM. The affinity resins were synthesized using a peptide coupling procedure to 6-aminohexanoic acid Sepharose **25** (Fig. 1). These solid supports produced a 20 to 40 fold improvement in transferase specific activity when eluted by a salt gradient in the presence of AdoMet. The NAT activity peak was well-separated from the major protein peak eluted by the gradient. Including AdoMet in the elution buffer stabilized the enzyme sufficiently to allow elution with the salt gradient. The elution profile of the transferase from columns formed from **16** and **17** was similar, but ligand **17** provided a greater separation from the main protein peak eluted by the gradient. The improved resolution is attributable to the additional recognition afforded by the methyl group. The prediction from the lower IC_{50} for **12** that NAT would bind more tightly to a column made from that ligand is borne out by the higher concentratation of salt required to elute the enzyme (0.9 M for **17** vs. 0.6 M for **16**, see Fig. 2).

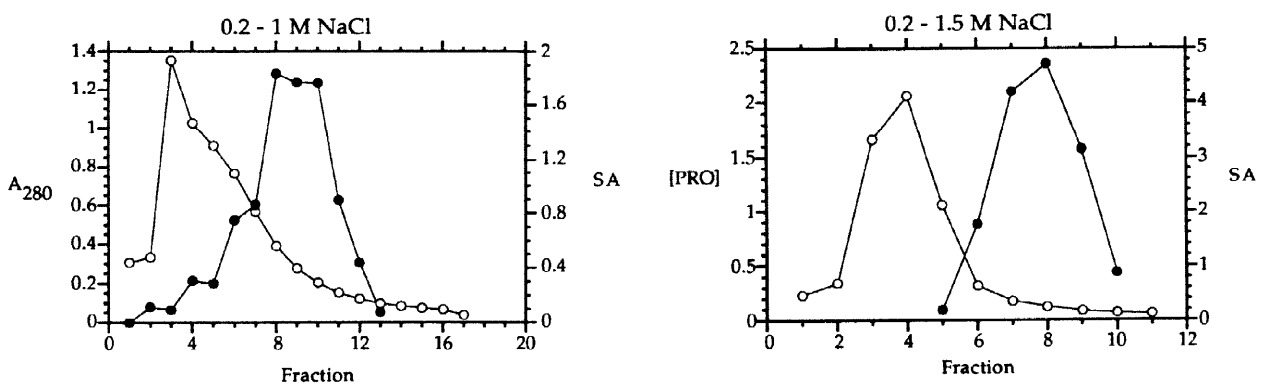


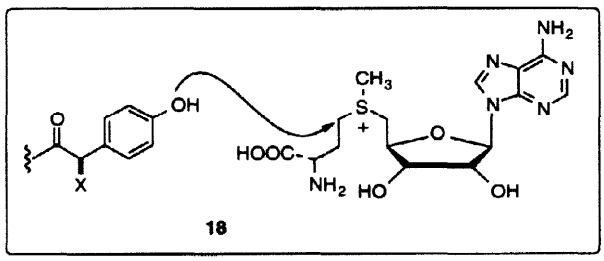
Figure 2. Elution profile of NAT and total protein from Sepharose-**16** (left) and Sepharose-**17** (right). The open circles represent protein concentration in mg/mL while the solid circles represent transferase specific activity in nmol isonocardicin A formed/min/mg of protein. The salt gradient for **16** was 0.2-1.0 M NaCl and 3 mL fractions were collected (peak activity at about 0.6 M NaCl). The salt gradient for **17** was 0.2-1.5 M NaCl and 5 mL fractions were collected (peak activity at about 0.9 M NaCl).

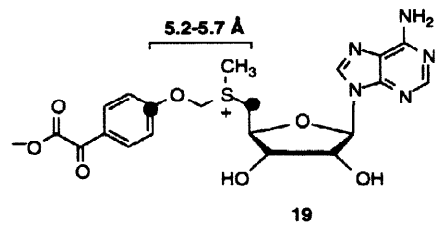
Although initially promising, these resins had several drawbacks. NAT activity was not recoverable in the absence of 0.25 M NaCl in the loading buffer. In fact, cellular proteins found in the dialyzed ammonium sulfate pellet adsorbed quantitatively to the resin in the absence of the high ionic strength loading buffer. This observation led to a postulate (that was supported by a control experiment with bovine serum albumin, which should not bind biospecifically) that ion exchange interactions were a major contributor to non-specific protein binding to the column. Ion exchange adsorption was detrimental to recovery of NAT activity, even in the presence of the stabilizing substrate AdoMet.

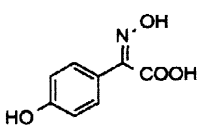
Researchers in the polyamine field have found similar non-specific binding and recovery problems with *S*-formycinylhomocysteine-Sepharose (**13**, see Scheme 3) used to purify AdoHcy/MTA nucleosidase. The enzyme required 1 M NaCl/4 mM MTA for elution and recovery was impossible if the protein solution loaded onto the column did not contain 0.2 M NaCl.[20] Spermidine synthase also required 0.3 M NaCl in the equilibration buffer for measurable recovery from decarboxylated AdoHcy-Sepharose (**14**, Scheme 3).[26] These ligands are positively charged at physiological pH and thus have the same inherent problems with non-specific ion exchange adsorption as the aminopropyladenosine derivatives **16** and **17**.

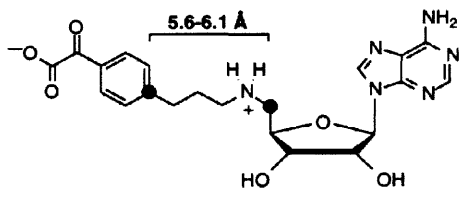
In an attempt to reduce the total number of proteins bound to the AdoMet analog affinity column, the three-dimensional structure of the ribose portion of the adenosyl moiety was changed to a morpholine ring **28** (Fig. 1) using the oxidation/reductive amination procedure of Ihara and Ishiwata.[33,34] Similar gels, 5'-AMP-[ox-red]-Sepharose and guanyl-(2',5')guanosine-[ox-red]-agarose, were found to be effective in the purification of Δ^2-IPP:AMP Δ^2-isopentenyl transferase and guanylribonuclease, respectively.[33,34] Utilizing the literature procedure, AdoHcy-[ox-red]-agarose was synthesized and the crude transferase preparation was loaded onto the gel in the presence or absence of salt (0.25 M NaCl). NAT did not bind to the column under either set of conditions. These results indicate that an intact ribose ring is necessary for recognition of the adenosyl moiety by the enzyme or that the spacer arm on the gel that occupies the 2' and 3' position is too sterically demanding for the enzyme to bind to the immobilized ligand.

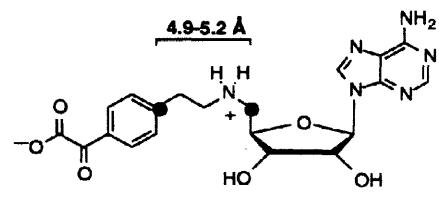
Scheme 5







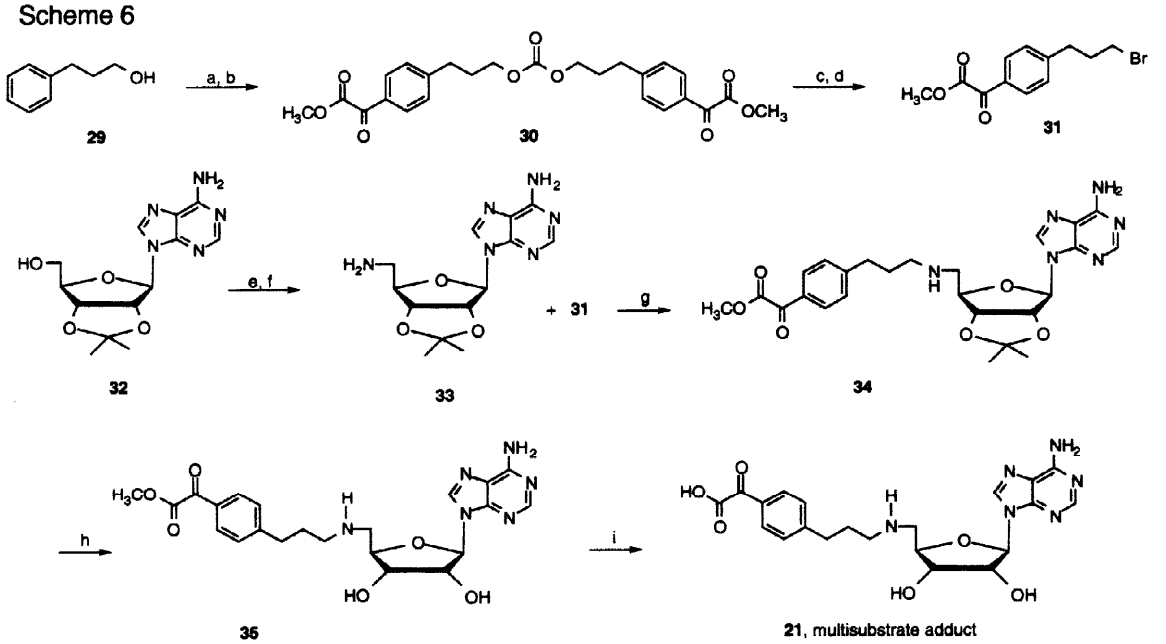




To potentially select for the transferase more efficiently than a simple AdoMet analog, a multisubstrate adduct affinity ligand **21** (Scheme 5) was designed.[29-31,35,36] Multisubstrate adduct inhibitors, by reducing the

entropic cost of binding two or more substrate molecules, can be potent enzyme inhibitors.[37] As a multisubstrate adduct incorporates key features of more than one substrate, the enzyme must naturally bind both substrates simultaneously in a ternary complex for it to bind a multisubstrate analog efficiently.[31] Kinetic[38] and stereochemical[13] evidence support this type of mechanism (BiBi) for NAT. It was hoped that by incorporating portions of the structural features for both the nocardicin substrate and AdoMet, the new ligand would be more selective for NAT than for other AdoMet-requiring enzymes. This approach has been adopted in designing inhibitors of catechol *O*-methyltransferase[36] and aminopropyl transferases.[19,22,29-31,36] Patterned after Coward's multisubstrate inhibitor of catechol *O*-methyltransferase,[36] the 3-amino-3-carboxypropyl side chain was eliminated. The electronic nature of the aromatic oxime of nocardicin E was mimicked with an aromatic α-ketoacid, which also provided the point of attachment to the solid support. A nitrogen was again substituted for sulfur at C-5' to ease synthetic manipulation and to increase stability in solution.[39] To approximate the presumed linear transition state generated by approach of the nocardicin *p*-hydroxyphenyl group on the S–CH_2 of AdoMet depicted in **18** (Scheme 5), the distance between C-5' (•) and the first aryl carbon (•) represented by the covalent adduct **19** was estimated in a molecular dynamics simulation (MSI Technologies, Insight II, version 970). When the energetically accessible conformations of **19** were explored, the distances between these two atoms were found to vary between 5.2-5.7 Å. The bisubstrate analogues **20** and **21** (Scheme 6) were compared in which an ammonium ion replaced the methylsulfonium in **19** and a two- or three-carbon linker connected this positive center to the aryl ring. As might be expected from the long C–S bonds in AdoMet, the two-carbon spacer underestimated the separation of the two sites (•) in **19**, 4.9–5.2 Å, whereas the three-carbon linker slightly overestimated the distance (5.6-6.1 Å, Scheme 5). Taking the view that the separation of the adenosyl C-5' and aryl carbon (•) would be greater in the actual transition state **18** than in **19**, and that the more flexible three-carbon analogue could more readily assume the correct conformation at the active site, **21** was selected for synthesis.

Scheme 6



Reagents: (a) NaH, carbonyldiimidazole, 89%; (b) $AlCl_3$, methyl oxalylchloride, 58%; (c) $NaOCH_3$, 90%; (d) CBr_4, PPh_3, 89%; (e) phthalimide, DEAD, PPh_3, 66%; (f) NH_2NH_2, 96%; (g) 3 equiv. of **24**, Et_3N, CH_3CN, reflux, 42%; (h) CF_3COOH, 81%; (i) NaOH, CH_3OH, 100%

Beginning with 3-phenyl-1-propanol **29**, the symmetrical carbonate was prepared from a reaction with NaH and carbonyldiimidazole (**Scheme 6**). Friedel-Crafts acylation[40] utilizing methyl oxalylchloride produced the di-substituted adduct **30** cleanly in 58% yield. Methanolysis of the carbonate afforded the corresponding alcohol which was converted to bromide **31** using the method of Hooz and Gilani.[41] 5'-Deoxy-5'-amino-2',3'-isopropylideneadenosine **33** was synthesized[32] from the commercially-available isopropylideneadenosine **32** by Mitsunobu reaction with phthalimide followed by hydrazinolysis in a total yield of 63%. Nucleophilic displacement[39] of bromide **31** by excess primary amine **33** afforded monosubstituted adduct **34**. Partial deprotection using aqueous trifluoroacetic acid provided the methyl ester **35** of the desired ligand in 80% yield. The deprotection was completed by saponification. The overall yield for the nine step convergent synthesis was 14% based on 3-phenyl-1-propanol.

Methyl ester **35** was tested as an inhibitor of the enzymatic reaction. The ester was used instead of the fully deprotected carboxylic acid **21** because once the ligand is immobilized on the column, this position becomes an amide; that is, the neutral ester matches the total charge on the immobilized ligand. The IC_{50} of **35** was 0.5 mM, slightly higher than **17**. The ligand was attached to aminohexyl agarose **24** with a peptide bond coupling procedure. Unfortunately, even in the best attempt, only 50% of the enzyme adhered to the resin. Once bound, either it could not be removed with AdoMet, AdoMet and 1 M NaCl, or 2 M NaCl, or activity was lost.

At this point, neutral AdoMet analog ligands were examined to eliminate ion exchange effects on the affinity column. Incorporating charge at the 5' position turned out to be detrimental to reproducibly good recovery of NAT activity. AdoHcy **15** was attached by a peptide bond to 6-aminohexanoic acid agarose **25**. Use of the gel with a carboxylic acid functionality (*vs.* an amine) also precluded anion exchange interactions with residual unmodified groups on the spacer arm.

Under the standard initial conditions for affinity chromatography, >90% of the total protein was not adsorbed, while NAT was quantitatively retained by AdoHcy-agarose. The recovery of activity was optimal when 1 mM AdoMet was used to elute NAT from the column, producing a protein solution increased in specific activity by 20-fold. This procedure is particularly appealing as it is a genuine biospecific affinity step: the enzyme binds to an immobilized inhibitor and is released and stabilized by the natural substrate. Although the degree of purification is not higher than that realized by the column made from **17**, this resin offers the important advantage of no added salt in the elution buffer. The transferase was the major protein at this point, contaminated by about 15 other proteins of lower concentration (Fig. 3, lane 2).

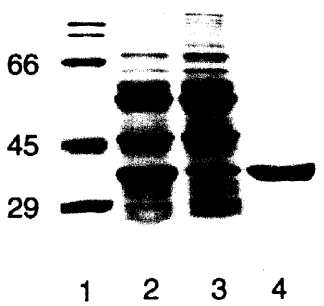


Figure 3. 10% SDS-PAGE analysis of the affinity purification of NAT. Lane 1 contains molecular weight markers. Lane 2 is the active protein fraction eluted from the AdoHcy-agarose column while lane 4 is the active fraction eluted from the nocardicin A-agarose column. Lane 3 is the flow-through of the nocardicin A-agarose column of protein not absorbed by this ligand.

A second affinity column was designed to take advantage of the difference between the transferase and other AdoMet-requiring enzymes: the binding site for nocardicin substrates. The first trial of this strategy

employed the simple oxime **22** (Scheme 5). This compound was synthesized in 49% overall yield from 4-hydroxy-α-oxobenzeneacetic acid in three steps. Protection of the carboxylic acid as a benzyhydryl ester was necessary to avoid decarboxylation in the subsequent step, formation of the oxime with hydroxylamine. A 4:1 *Z*:*E* mixture of oxime isomers resulted. The benzhydryl group was cleaved with acid to yield **22.** The oxime was attached to Affi-Gel **26**, an agarose matrix with a hydrophilic, peptide-like spacer arm. AffiGel was chosen to mimic the polarity of the peptide bonds of the nocardicins and, in one possible conformation, the spacer arm could mimic the β-lactam. NAT, unfortunately, did not bind to this column under any of the conditions attempted. This poor binding could owe to steric inaccesibility: utilizing the spacer arm as part of the recognition site effectively shortens the arm and puts the enzyme closer to the physical boundary of the gel surface. These results are supported by the fact that NAT would not accept di- or tri-peptides as substrates and these peptides did not inhibit the normal reaction, even at 2 mM concentration.[42]

From these results, it was proposed that a closer match to the nocardicin site, especially with regard to the strained geometry of the β-lactam, was required than was provided by the much smaller oxime. Nocardicins E, F, and G were not candidates for immobilization owing to the synthetic challenge of their preparation[43] and the quantity necessary for column synthesis. Nocardicin A was chosen because it was available in sufficient quantity from isolation. A potential problem with this column was the possibility that binding could be quite tenacious: the $K_{m(app)}$ of NAT for nocardicin E is 14 μM.[38] Nocardicin A was coupled by the carbodiimide method first to aminohexylagarose **24**, forming a peptide bond with carboxylic acid functionalities at C-10' and C-10 of **1**. The two regioisomeric sites of attachment were not a cause for concern; mimicking as large a binding site as the one for nocardicin E requires incorporating many steric constraints, and giving NAT a choice of binding sites increased the chances that the column would retain the enzyme. It was known from the AdoMet analogs that attachment by the amino acid terminus preserved binding, so to the extent that the resin was represented by that coupling orientation, it was hoped that NAT would bind. NAT did bind to this resin, but was inactivated. This outcome presumably owes to the residual amino groups that cause ion exchange. In the second attempt, nocardicin A was coupled to ω-aminohexanoic acid agararose **25**, forming a peptide bond with the amino group of C-9', a single point of attachment with no residual anion exchange. However, this matrix was also ineffective as a purification tool for NAT owing to poor recovery. In contrast, when nocardicin A was attached by the bromoacetyl method (Fig. 1), the resulting gel was successful in the purification of the transferase. The binding of NAT to the amide-linked nocardicin columns was effectively irreversible owing to the size of the ligand and the exact steric match between the immobilized antibiotic and one of the substrates, nocardicin E. Many hydrogen bonds and hydrophobic interaction sites are possible. If the phenol acts as the nucleophile in the coupling reaction (pH 9), the spacer arm on the gel occupies part of the nocardicin binding site. This steric bulk could decrease the affinity of the transferase for the nocardicin column sufficiently for the elution to occur.

The active fraction from AdoHcy-agarose was loaded directly on to the nocardicin-agarose column. In the trial shown, the nocardicin A affinity column was slightly overloaded and a small amount of NAT was visible in the flow through (Fig. 3, lane 3). Again, elution was optimal using a stepwise procedure with buffer containing 1 mM nocardicin A, 1 mM AdoMet, and 1 M NaCl. This protein solution was homogeneous by SDS-PAGE, giving a single band at about 38 kDa (Fig. 3, lane 4).

Discussion

The nocardicin A-agarose column resulted in only a 4-fold increase in the specific activity of NAT from the first affinity step, but the protein was homogeneous on even a silver-stained SDS-PAGE gel (data not shown). Whatever inactivation of the enzyme may have occurred on exposure to 1 M salt at this point in the purification seems to have been minimal judging from its relative proportion in lane 2 (Fig. 3). Perhaps the presence of product and AdoMet in the elution buffer more than compensated for the destabilizing effects of ionic strength. Notwithstanding, for the purpose of primary sequence analysis of both the *N*-terminal region, and proteolytic fragments, this was a more than sufficient outcome and has allowed its encoding gene to be identfied and cloned.[38]

Table 1. Summary of affinity ligands screened and their behavior in the purification of NAT.

Compound	IC_{50}	Useful Affinity Ligand?
AdoHcy, **15**	1 mM	yes, as long as coupling strategy is optimized
16	1 mM	no, charge at 5' limits recovery
17	0.4 mM	no, charge at 5' limits recovery
bisubstrate, **21**	0.5 mM	no, charge at 5' limits recovery
oxime, **22**	ND	no, binding is poor, possibly for steric reasons
nocardicin A, **1**	ND	yes, as long as coupling strategy is optimized

The importance of ion exchange effects underlies all aspects of the purification of NAT and was not fully appreciated in the development of affinity matrices. The transferase was observed to be unusually sensitive to these effects, a factor that severely limited experimental options to be used in the purification. The lessons learned are broadly summarized in Table 1. Three conclusions may be drawn. First, either through the introduction of charged ligands, or through the *residual* charged groups unreacted on the resin itself (coupling is often <50% efficient), ion exchange interactions between the matrix and the protein will occur. For the case of NAT, these proved largely detrimental. Second, a potential ligand can be tested for inhibition of the normal reaction. Concentrations that give an IC_{50} on the order of 1 mM are fully sufficient to separate the protein of choice and yet not bind it so tightly that drastic conditions are required for its release. Ideally, such a ligand will give competitive or non-competitive inhibition against one (or more) substrates suggesting specifity for the enzyme of interest. For example, AdoHcy **15** *vs.* AdoMet shows a competitive inhibition pattern with NAT. Third, several, if not all, possible means of attachment of the ligand to a solid support should be screened to empirically determine the most practically efficient. Sites of ligand attachment can also be important for active site recognition. Sampling various linkage strategies, as was seen in the ultimately successful construction of a nocardicin A affinity gel, can leave these critical functions free but anchor the ligand at a relatively less important locus.

Experimental Section

Materials and Methods

Anhydrous reactions were carried out in oven- or flame-dried glassware under positive nitrogen pressure. Tetrahydrofuran was distilled from sodium benzophenone ketyl immediately prior to use.

Methylene chloride was distilled from P_2O_5 or CaH_2 immediately prior to use. Acetonitrile was distilled from CaH_2 and used immediately or stored in oven-dried hypovials over 4 Å molecular sieves. Triethylamine was distilled from CaH_2 and stored in 15 mL aliquots in dry hypovials. Dimethylformamide was distilled from CaH_2 and stored over 4 Å molecular sieves or sequentially dried over 4 Å molecular sieves.

Analytical thin-layer chromatography was performed on Analtech Uniplate glass plates coated with silica gel (0.25 mm) containing fluorescent indicator. Flash chromatography utilized Merck Kieselgel 60 and the mobile phase indicated in individual procedures.

Melting points were determined with a Thomas-Hoover oil bath apparatus in open capillaries and are uncorrected. Infrared spectra were obtained using a Perkin-Elmer 1600 Series FTIR spectrophotometer. Proton NMR spectra were recorded using a Varian XL/VXR-400 operating at 400 MHz or a Brucker AMX-300 operating at 300 MHz. Chemical shifts are reported in ppm downfield from tetramethylsilane or 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS) as internal standards. As appropriate, spectra were also referenced to $CHCl_3$ at 7.26 ppm, methanol at 3.30 ppm, or acetone at 2.04 ppm. Aqueous NMR samples were also referenced to acetone at 2.22 ppm when recovery was desired. Coupling constants are reported in Hz and peak multiplicities described as singlet (s), doublet (d), triplet (t), multiplet (m), or broad (br). ^{13}C $\{^1H\}$ NMR spectra were recorded on a Brucker AMX-300 operating at 75 MHz. Chemical shifts are reported referenced to $CDCl_3$ (solvent) at 77.0 ppm or, for aqueous samples, to *p*-dioxane at 66.7 ppm as an internal standard. High and low resolution mass spectra were obtained on a VG Instruments 70-S GC/MS at 70 eV in either the EI^+ or CI^+ (NH_3) operating mode.

Synthesis of Oxime 22.

Diphenylmethyl 4-hydroxy-α-oxo-benzeneacetate. 4-Hydroxy-α-oxo-benzeneacetic acid (0.47 g, 2.83 mmol) was dissolved in 5 mL of ethyl acetate. Diphenyldiazomethane[44] (0.60 g, 3.11 mmol, 1.1 equiv) in 5 mL of ethyl acetate was added dropwise, and the purple solution was stirrred at room temperature for 15 min. The mixture was evaporated and purified on 30 g of silica eluted with 30% ethyl acetate in hexane (R_f 0.42). Two very small high R_f spots were visible, so the oily residue was repurified on 30 g of silica gel after preadsorbing on 2 g of silica gel. Elution with 20% ethyl acetate in hexane provided the product (810 mg, 93%) as a yellow oil, R_f 0.27, which was used without further purification. NMR (300 MHz, CD_2Cl_2) δ = 7.84 (d, J = 8.8 Hz, 2H); 7.45-7.31 (m, 10 H); 7.09 (s, 1H, $CHPh_2$); 6.90 (d, J = 8.8 Hz, 2H); 6.2 (br, 1H, OH), IR ($CHCl_3$) 3574, 3366, 3026, 1729, 1680, 1598, 1581, 1280, 1198, 1159, 989.

Diphenylmethyl 4-Hydroxy-α-hydroxyimino-benzeneacetate. The benzhydryl ester above (552 mg, 1.79 mmol) in 10 mL of absolute ethanol was added slowly to a solution of hydroxylamine hydrochloride (195 mg, 2.85 mmol, 1.5 equiv) and pyridine (460 mL, 5.7 mmol, 3 equiv) in 5 mL of EtOH. The solution was stirred at room temperature for 18 h, evaporated and the residue taken up in ethyl acetate. The organic layer was washed with water and brine, dried over anhydrous sodium sulfate, and evaporated. The residue was purified by flash chromatography on 30 g of silica gel after preadsorption on 3 g of silica. Elution with ethyl acetate:hexane, 1:2 (R_f 0.36) yielded 419.5 mg (72%) of pure product as a white powder, mp 115-117 °C. NMR spectroscopy showed a 4:1 *Z:E* mixture of isomers. The major isomer was assumed to be the hydrogen bonded *Z* -isomer and was assigned in analogy to values for nocardicin E and nocardicin F.[43,45] NMR (300 MHz, d_6 acetone) δ = 10.85 (s, 0.8H, H-bonded oxime OH, *Z*-isomer); 8.95 (s, 1H, phenol OH); 8.85 (s, 0.2H, oxime OH, *E* isomer); 7.50 (d, J = 8.8 Hz, 2H); 7.45-7.32 (m); 7.19 (s, 0.8H, benzhydryl CH, *Z*-oxime); 7.04 (s,

0.2H, benzhydryl CH, *E*-oxime); 6.87 (d, J = 8.6 Hz, 0.4H); 6.83 (d, J = 8.6 Hz, 1.6H), ^{13}C {^1H} NMR (d_4 methanol) δ = 165.4, 160.4, 151.6, 140.9, 132.2, 129.4, 128.3, 127.9, 123.2, 116.5, 79.4 (only *Z*-isomer visible except for two minor peaks at 129.1 and 128.4 which can be assigned to the benzhydryl group on the *E*-isomer), IR ($CHCl_3$) 3581, 1736, 1605, 1516, 1224, 1210, 1170, Anal. calculated for $C_{21}H_{17}NO_4$: C, H, N.

4-Hydroxy-α-hydroxyimino-benzeneacetic acid, 22. The benzhydryl oxime (680 mg, 1.96 mmol) was dissolved in a minimum amount of CH_2Cl_2 and cooled to 0 °C. Precooled TFA (trifluoroacetic acid, 10 mL) was added and the mixture stirred at 0 °C for 30 min then allowed to warm to room temperature. The orange solution was evaporated. The residue was resuspended in CH_2Cl_2, then coevaporated with ethyl acetate and toluene to a yellow powder/oil mixture that was triturated extensively with CH_2Cl_2 and filtered. The resulting tan powder, 173.1 mg (49%), was dried in vacuo. mp 155-157 °C, lit.[46] mp 156 °C. NMR (400 MHz, d_6 DMSO) δ = 12.0 (br, oxime OH); 9.75 (s, phenol OH); 7.40 (d, J = 8.8 Hz, 2H); 6.80 (d, J = 8.8 Hz, 2H).

Synthesis of Multisubstrate Analogue, 21.

Bis-3-phenylpropylcarbonate. Sodium hydride (660 mg of 80% NaH, 22 mmol, 1 equiv.) was washed free of oil with pentane. About 15 mL of freshly distilled THF was added and the suspension was cooled to 0 °C. Neat 3-phenyl-1-propanol **29** (3.0 mL, 22.0 mmol) was added dropwise. After evolution of hydrogen was complete, carbonyldiimidazole (1.96 g, 12.1 mmol, 0.55 equiv) was added as a solution in a few mL of dry DMF. The solution was stirred for 30 min at 0 °C and then poured into water and extracted with ethyl acetate. The combined organic layers were extracted with 1 N HCl, water, and 5% $NaHCO_3$; washed with brine; dried over anhydrous $MgSO_4$; and evaporated, yielding 3.46 g of crude product, which was preadsorbed on to silica gel. Purification by filtering through 50 g of silica gel eluting with 10% ethyl acetate/hexane yielded 2.91 g (89%) of the product as a colorless oil. R_f 0.42 (10% ethyl acetate/hexane), 0.11 (2% ethyl acetate/hexane). NMR (300 MHz, $CDCl_3$) δ = 7.25 (m, 10 H); 4.16 (t, J = 6.5 Hz, 4 H); 2.73 (t, J = 7.8 Hz, 4H); 2.02 (m, 4H), MS (CI, NH_3) m/e 316 (M + 18), Anal. calculated for $C_{19}H_{22}O_3$: C, H.

[3-[4'-(Methyl)-2-oxoacetyl]phenyl]propylcarbonate, 30. A modification of the procedure of Julia [40] was used. Aluminum chloride (6.16 g, 46 mmol, 5.0 equiv) was suspended in 50 mL of freshly distilled methylene chloride. After cooling to 0 °C, methyl oxalyl chloride (4.98 mL, 46 mmol, 5.0 equiv) was added via syringe and a pale yellow solution was obtained. The carbonate above (2.76 g, 9.25 mmol) was dissolved in about 5 mL of freshly distilled methylene chloride and added to the flask dropwise via syringe. The reaction mixture was stirred overnight at room temperature. The orange suspension was rinsed into $CHCl_3$/1 N HCl in a 1 L separatory funnel. The organic layer was collected and the aqueous layer was extracted with $CHCl_3$ (vent carefully). The combined organic layers were washed with 1 N HCl, water, and brine; dried over anhydrous Na_2SO_4; and evaporated to 5.6 g of residue. The product was purified by flash chromatography on 100 g of silica gel (preadsorbed on 10 g of silica gel) eluted with 30% ethyl acetate/hexane. The title compound, 2.54 g (58%), was recovered as a yellowish oil, R_f 0.10 (20% ethyl acetate/hexane), 0.53 (50% ethyl acetate/hexane). NMR (300 MHz, $CDCl_3$) δ = 7.94 (d, J = 8.3 Hz, 4H); 7.33 (d, J = 8.2Hz, 4H); 4.16 (t, J = 6.4 Hz, 4H); 3.97 (s, 6H); 2.80 (t, J = 7.7 Hz, 4H); 2.03 (m, 4H), ^{13}C {^1H} NMR ($CDCl_3$) δ = 185.4, 163.9, 154.8, 148.9, 134.9, 130.1, 128.8, 66.7, 52.4, 31.9, 29.4. IR ($CHCl_3$) 3030, 2960, 1738, 1687, 1608, 1265, 1170, 1004, MS (CI, NH_3) m/e 488 (M+18), Anal. calculated for $C_{25}H_{26}O_9$: C, H.

Methyl [4-(3-hydroxypropyl)]-α-oxobenzene acetate. About 5 mL of dry methanol and 5 mL of freshly distilled methylene chloride was added to the Friedel-Crafts reaction product **30** (2.29 g, 4.86 mmol) followed

by $NaOCH_3$ dropwise (1.10 mL of 25% solution, 4.86 mmol, 1.0 equiv). The clear, colorless solution was stirred at room temperature for 4 hours. Acetic acid (2.5 mL, excess) was added to quench the base, and the reaction mixture was evaporated onto 10 g of silica gel. Purification of the alcohol was conducted by flash chromatography, 100 g of silica gel, eluted with 25% ethyl acetate/hexane. The title compound was recovered as a colorless oil, 1.94 g (90%), R_f 0.28 (50% ethyl acetate/hexane). NMR (300 MHz, $CDCl_3$) δ = 7.94 (d, J = 8.4 Hz, 2H); 7.33 (d, J = 8.4 Hz, 2H); 3.97 (s, 3H); 3.68 (t, J = 6.3 Hz, 2H); 2.80 (t, J = 7.7 Hz, 2H); 1.90 (m, 2H), ^{13}C {^1H} NMR ($CDCl_3$) δ = 185.6, 164.1, 150.2, 135.2, 130.1, 128.9, 61.5, 52.6, 33.4, 32.1, IR ($CHCl_3$) 3613, 3025, 2950, 2884, 1737, 1684, 1607, 1320, 1226, 1173, 1044, 1008.

Methyl [4-(3-bromopropyl)]-α–oxobenzeneacetate, 31. The alcohol from methanolysis of the carbonate (251 mg, 1.17 mmol) was dissolved in about 7 mL of freshly distilled methylene chloride and the solution was cooled to 0 °C. Carbon tetrabromide (390 mg, 1.17 mmol, 1 equiv) and triphenylphosphine (460 mg, 1.76 mmol, 1.5 equiv) were added in one portion each. The solution was stirred at 0 °C for 30 min. The reaction mixture was then evaporated onto 1.5 g of silica gel and purified by flash chromatography (15 g of silica gel, eluted with 10% ethyl acetate/hexane) to yield the bromide (298 mg, 89%) as a slightly yellow oil. R_f 0.47 (25% ethyl acetate/hexane), 0.28 (10% ethyl acetate/hexane). NMR (300 MHz, $CDCl_3$) δ = 7.96 (d, J = 8.4 Hz, 2H); 7.34 (d, J = 8.4 Hz, 2H); 3.98 (s, 3H); 3.39 (t, J = 6.5 Hz, 2H); 2.88 (t, J = 7.4 Hz, 2H); 2.19 (m, 2H), ^{13}C {^1H} NMR ($CDCl_3$) δ = 185.2, 163.7, 148.3, 130.1, 129.8, 128.7, 52.2, 33.6, 33.0, 32.4, MS (CI, NH_3) m/e 302, 304 (M + 18), Anal. calculated for $C_{12}H_{13}BrO_3$: C, H, Br.

5'-(1,3-Dihydro-1,3-dioxo-2H-isoindol-2-yl)-2',3'-(1-methylethylidene)-5'-deoxyadenosine.[32]
2',3'-Isopropylidene adenosine **32** (Aldrich, 8.82 g, 28.7 mmol), phthalimide (4.34 g, 29.5 mmol, 1.03 equiv), and triphenylphosphine (7.52 g, 28.7 mmol, 1 equiv) were suspended in 100 mL of freshly distilled THF. Diethylazodicarboxylate (5.0 g, 28.7 mmol, 1 equiv) was added dropwise. The suspension became an orange solution with generation of heat. After 15 min, a precipitate appeared. After stirring for 2 h, the suspension was filtered and the precipitate was washed extensively with ether and dried in vacuo to yield 8.26 g (66%) of **32** as an off-white solid. Recrystallization from acetone/ether afforded colorless leaves, mp 142-144 °C (lit.[32] mp 198 °C). NMR (300 MHz, $CDCl_3$) δ = 8.06 (s, 1H); 7.87 (s, 1H); 7.80-7.68 (m, 4H); 6.03 (d, J = 1.8 Hz, 1H); 5.64 (br s, 2H, adenine NH_2); 5.52 (d x d, J = 1.8, 6.4 Hz, 1H); 5.24 (d x d, J = 3.5, 6.3 Hz, 1H); 4.54 (m, 1H); 4.01 (8 lines; J_{AB} = 14.1, J_{AX} = 6.0, J_{BX} = 6.2 Hz, 2H); 1.58 (s, 3H); 1.37 (s, 3H).

5'-Deoxy-5'-amino-2',3'-(1-methylethylidene)adenosine, 33.[32] 5'-Deoxy-5'-phthaloyladenosine (7.60 g, 17.4 mmol) was suspended in 500 mL of ethanol. Hydrazine hydrate (15 mL, 280 mmol) was added and the flask equipped with a reflux condenser. After refluxing for 2 h, TLC (10% $CH_3OH/CHCl_3$) showed the reaction was complete. After cooling to room temperature, the suspension was filtered. The filtrate was evaporated; the residue was taken up in 60 mL of deionized H_2O and the pH adjusted to 4 with glacial acetic acid. After storing at 4 °C for 4 hours, the mixture was filtered. The filtrate was brought to pH 10 with 10 N NaOH, transferred to a 1 L separatory funnel, and extracted with 4 x 150 mL of $CHCl_3$. The combined organic layers were dried over anhydrous sodium sulfate, evaporated, and dried in vacuo. The resulting nearly white powder was triturated with 3 x 100 mL of ether to yield 5.16 g (96%) of **33** as a white powder, mp 204-205 °C (dec), (lit.[32] mp 203 °C). NMR (300 MHz, $CDCl_3$) δ = 8.34 (s, 1H); 7.92 (s, 1H); 6.03 (d, J = 3.08 Hz, 1H); 5.73 (br s, 2H, adenine NH_2); 5.46 (d x d, J = 3.1, 4.5 Hz, 1H); 5.02 (d x d, J = 3.5, 6.5 Hz, 1H); 4.25 (m, 1H); 2.92 (8 lines, J_{AB} = 13.4 Hz, J_{AX} = 5.9 Hz, J_{BX} = 4.5 Hz, 2H); 1.62 (s, 3H); 1.39 (s, 3H).

Protected multisubstrateadduct ligand: [4-(3-{[6-(6-aminopurin-9-yl)-2,2-dimethyl-tetrahydro-furo[3,4-d][1,3]dioxol-4-ylmethyl]-amino}-propyl)-phenyl]-oxo-acetic acid methyl ester, 34. 5'-Aminoadenosine **33** (1.01 g, 3.3 mmol, 3 equiv) was suspended in 30 mL of dry acetonitrile. Triethylamine (440 mL, 3.3 mmol, 3 equiv) was added dropwise followed by bromide **31** (297.3 mg, 1.04 mmol) as a solution in 3 mL of acetonitrile. The mixture was heated to 70 °C and maintained overnight. After cooling to room temperature, the acetonitrile was evaporated. The foamy residue was taken up in chloroform, washed with 0.1 M Na_2CO_3, dried over anhydrous Na_2SO_4, and evaporated to 1.2 g crude material. The product was recovered as a white foam (224 mg, 42%) from 50 g of silica gel eluted with 5% CH_3OH/CH_2Cl_2. R_f 0.29 (10% $CH_3OH/CHCl_3$). NMR (300 MHz, $CDCl_3$) δ = 8.32 (s, 1H); 7.89 (s, 1H); 7.92 (d, *J* = 8.3 Hz, 2H); 7.28 (d, *J* = 8.3 Hz, 2H); 6.00 (d, *J* = 3.1 Hz, 1H); 5.64 (br s, 2H, NH_2); 5.48 (d x d, *J* = 3.1, 6.4 Hz, 1H); 5.02 (d x d, *J* = 3.3, 6.4 Hz, 1H); 4.35 (m, 1H); 3.97 (s, 3H); 2.89 (app t, *J* = 4.7 Hz, 2H); 2.73-2.58 (m, 4H); 1.79 (m, 2H); 1.61 (s, 3H); 1.38 (s, 3H), ^{13}C {^1H} NMR ($CDCl_3$) δ = 185.4, 164, 155.8, 152.8, 150.2, 149.1, 139.7, 130.1, 130.07, 128.7, 120.1, 114.3, 90.6, 85.4, 93.2, 52.5, 51.1, 48.8, 33.5, 30.7, 27.1, 25.2, IR ($CHCl_3$) 3526, 3414, 2993, 1740, 1684, 1631, 1606, 1472, 1418, 1375, 1328, 1172, 1078, 1007, Anal. calculated for $C_{25}H_{30}N_6O_6 \bullet H_2O$: C, H, N.

Partially deprotected multisubstrate adduct ligand, 35. Protected mutisubstrate adduct ligand **34** (18 mg, 0.034 mmol) was dissolved in 1.5 mL of TFA and 150 μL of H_2O. After stirring for 1 hr, the solution was evaporated and coevaporated with toluene to remove the TFA. The oily residue was triturated with ether to produce a white powder which was dried in vacuo. The partially deprotected ligand was recovered as a trifluoroacetate salt (19 mg, 81% for di-TFA salt). NMR (300 MHz, D_2O) δ = 8.34 (s, 1H); 8.30 (s, 1H); 7.79 (d, *J* = 8.3 Hz, 2H); 7.28 (d, *J* = 8.3 Hz, 2H); 6.04 (d, *J* = 4.6 Hz, 1H); 4.38 (m, 2H, C-2' + C-3'); 4.35 (m, 1H); 3.96 (s, 3H); 3.52 (m, 2H, C-5'); 3.09 (t, *J* = 8.1 Hz, 2H); 2.73 (t, *J* = 7.4 Hz, 2H); 1.99 (m, 2H).

Multisubstrate adduct ligand, 21. The di-trifluoroacetate salt **35** (77 mg, 0.11 mmol) was taken up in 4 mL deionized H_2O and 1 mL CH_3OH. NaOH (1 N solution, 350 μL, 3.1 equiv) was added. The mixture was stirred at room temperature for 30 min, neutralized, and lyophilized to yield 84.6 mg of product which contained 0.22 mmol (23 mg) of sodium trifluoroacetate. The yield of ligand, therefore, was 62 mg (100%) as white solid. NMR (300 MHz, D_2O) δ = 8.16 (s, 1H); 8.07 (s, 1H); 7.74 (d, *J* = 8.3 Hz, 2H); 7.22 (d, *J* = 8.3 Hz, 2H); 5.99 (d, *J* = 5.2 Hz, 1H); 4.82 (m, C-2', partially obscured by the HDO peak); 4.41 (m, 2H, C-3' + C-4'); 4.35 (m, 1H); 3.49 (m, 2H, C-5'); 3.10 (t, *J* = 7.7 Hz, 2H); (2.69 (t, *J* = 7.5 Hz, 2H); 1.95 (m, 2H).

General Syntheses of Affinity Columns

Bromoacetyl method.

Bromoacetyl *N*-hydroxysuccinimide. *N*-Hydroxysuccinimide (276 mg, 2.4 mmol, 1.2 equiv.), bromoacetic acid (278 mg, 2.0 mmol) and dicyclohexylcarbodiimide (454 mg, 2.2 mmol, 1.1 equiv.) were added to 16 mL of *p*-dioxane. After stirring 1 h at room temperature, the suspension was filtered and the filtrate was evaporated to dryness. The residue was crystallized from CH_2Cl_2 and petroleum ether to yield 452 mg (96%) of the known active ester as white needles.[38]

Bromoacetyl Aminohexylagarose. About 250 mg (1.2 mmol) of active ester was dissolved in 3 mL of *p*-dioxane and added to 5 mL of ω-aminohexylagarose (Sigma, 5-10 μmol of amino groups per mL of gel) pre-equilibrated in 0.1 M sodium phosphate, pH 7.5. After standing for 45 min at 4 °C, the gel was washed with 1 L of 0.1 M NaCl and 200 mL of 0.1 M $NaHCO_3$, pH 9.0.

Coupling reaction. The ligand of interest (0.1 mmol) was dissolved in 2.5 mL of 0.1 M $NaHCO_3$, pH 9.0 and added to bromoacetyl aminohexylagaraose. After incubating 3 days at room temperature, the gel was washed with 1 L of 0.2 M NaCl and 200 mL of 0.1 M $NaHCO_3$, pH 9.0 and placed in a 50 mL filter flask. After degassing under vacuum, the suspension was reacted with 5.0 mL of 0.2 M β-mercaptoethanol in the same buffer. The column was washed with 1 L of 0.2 M NaCl and stored in the presence of 0.4 % NaN_3 at 4 °C until needed.

Carbodiimide method (for ligands with amino groups).

6-Aminohexanoic acid Sepharose 4B (Sigma, 4 mL, containing 10-14 μmol of 6-aminohexanoic acid per mL of gel) was washed with 500 mL of 0.5 M NaCl and 500 mL of deionized water. The ligand (0.5 mmol) was added as a solution in 2.0 mL of deioinzed water followed by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (200 mg, 1 mmol), dropwise, as a solution in 6.0 mL of deionized water. The pH of the suspension was adjusted to 4.5 and monitored periodically for about 1 h, adjusting again as necessary. At that time, the gel suspension was covered and allowed to stand at room temperature overnight. The gel was washed with 500 mL of 0.5 M NaCl and stored in the presence of 0.4 % NaN_3 at 4 °C until needed.

Carbodiimide method (for ligands with carboxylic acid groups).

Aminohexyl Sepharose 4B (Sigma, 4 mL, containing 6-10 μmol of amino groups acid per mL of gel) was washed with 500 mL of 0.5 M NaCl and 500 mL of deionized water. The ligand (1 mmol) was added as a solution in 3.0 mL of deioinzed water followed by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (400 mg, 2 mmol), dropwise, as a solution in 3.0 mL of deionized water. The pH of the suspension was adjusted to 4.5-6 and monitored periodically for about 1 h, adjusting again as necessary. At that time, the gel suspension was covered and allowed to stand at room temperature overnight. The gel was washed with 500 mL of 0.5 M NaCl and stored in the presence of 0.4 % NaN_3 at 4 °C until needed.

Oxidation-reduction method (AdoHcy-[ox-red]-Agarose).

ω-Aminohexylagarose (5 mL, 7.5 μmol of amino groups per mL of gel) was washed with 100 mL of 0.5 M NaCl, 500 mL of deionized water, and 200 mL of 0.1 M $Na_2B_4O_7$, pH 9.0. The ligand (0.13 mmol) was dissolved in 1.5 mL of deionized water and added to the gel at 0 °C. $NaIO_4$ (28 mg, 0.13 mmol) was added as a solution in 0.5 mL of deionized water. After standing 1 hr at 0 °C, the pH of the yellow suspension was adjusted to 9.0 with saturated $Na_2B_4O_7$ and the mixture was kept overnight at 4 °C. Sodium borohydride (5.0 mg, 0.13 mmol) was added to the gel suspension as a solution in 0.5 mL of 0.1 M $Na_2B_4O_7$, pH 9.0. After 2 hrs at 4 °C, the gel was washed with 100 mL of deionized water, 500 mL of 0.1 M $Na_2B_4O_7$, pH 9.0, 500 mL of 4 M NaCl, and 500 mL of deioized water. The column was stored in the presence of 0.4 % NaN_3 at 4 °C until needed.

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