Bisubstrate Ketone Analogues as Serotonin *N*-Acetyltransferase Inhibitors

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Serotonin *N*-acetyltransferase, also called the melatonin rhythm enzyme, is thought to play an important regulatory role in circadian rhythm in animals and people. A series of analogues were synthesized in which indole and coenzyme A were linked via ketone tethers as designed inhibitors of this enzyme. These compounds were tested against purified serotonin *N*-acetyltransferase. The parent ketone compound was found to be as potent as an amide linked compound studied previously, suggesting that there are no key hydrogen bonds to the nitrogen atom of the corresponding substrate necessary for tight inhibition. Reduction of the parent ketone afforded the diastereomeric carbinol mixture which showed reduced inhibitory potency, arguing against tetrahedral analogue mimicry as an important inhibitory theme. Several conformationally constrained ketone analogues were synthesized and investigated, and the results indicated that directing the orientation of the two substrates within the bisubstrate system could be used to maximize enzyme inhibition.

Serotonin *N*-acetyltransferase (arylalkylamine *N*-acetyltransferase, AANAT) is the light-regulated enzyme responsible for the diurnal variation in melatonin production.[1] It serves as an attractive inhibitory target for the development of chemical tools designed to reveal the biological roles of melatonin in the regulation of circadian rhythm. Such enzyme inhibitors might ultimately have applications in the treatment of sleep and mood disorders. A previous effort to develop inhibitors of this enzyme started with consideration of the enzyme mechanism. AANAT catalyzes the transfer of the acetyl group from acetyl-CoA to serotonin (**1a**) and the alternative substrate tryptamine (**1b**) (see Scheme 1) via an ordered substrate binding, ternary complex kinetic mechanism.[2] It was therefore reasonable to assume that bisubstrate analogue inhibitors such as **2** in which tryptamine was linked to CoA would result in potent inhibition. This expectation was realized in that tryptamine linked to CoA via an acetyl bridge proved to be an effective inhibitor against AANAT, with a K_i value about 1000-fold lower than the substrate K_m values.[3] This inhibitor **2** was competitive versus acetyl-CoA and noncompetitive versus tryptamine and also exhibited a slow off-rate.[3,4] A cocrystal structure of the compound bound to AANAT showed that both CoA and indole moieties were interacting with the enzyme active site and revealed several key contacts involved in stabilizing the interaction and the catalytic mechanism.[5,6] This structure has served as a model for other so-called GNAT (general control nonrepressed factor 5 related acetyltransferases) superfamily members.[7] A number of other bisubstrate analogues with substitutions in the indole, CoA, and linker moiety were prepared and evaluated as AANAT inhibitors. These studies revealed that a methylene extension of the linker (propionyl) (**3**) led to an inhibitor that was about as potent as the parent compound **2** and that inclusion of an extra methyl group in the linker (isopropionyl, **4**) led to even more potent inhibition (Figure 1).[4]

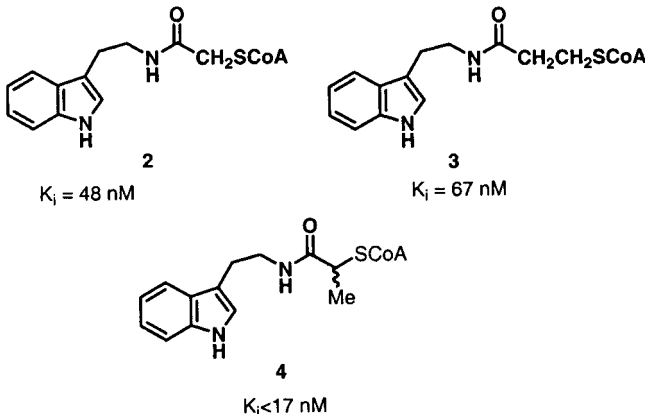
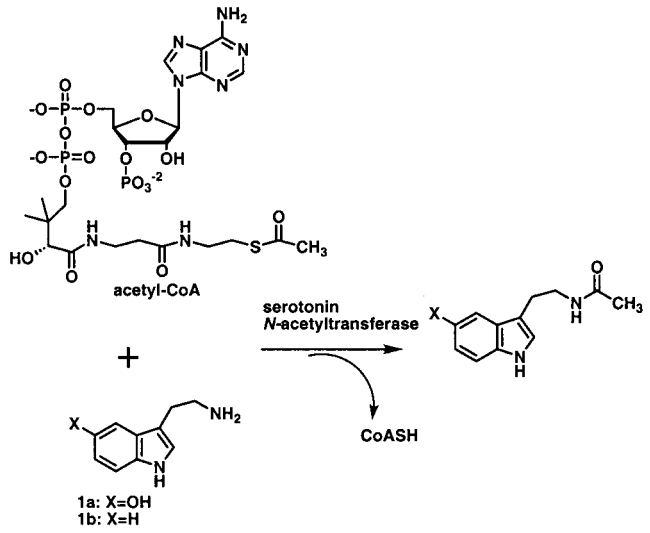


Figure 1. Previously prepared bisubstrate inhibitors of AANAT.

Scheme 1. AANAT Catalyzed Reaction



In this study, we have prepared a series of novel bisubstrate analogues that allow a more detailed understanding of the role of the linking region in AANAT inhibition. A conformationally restrained analogue (**8j**)

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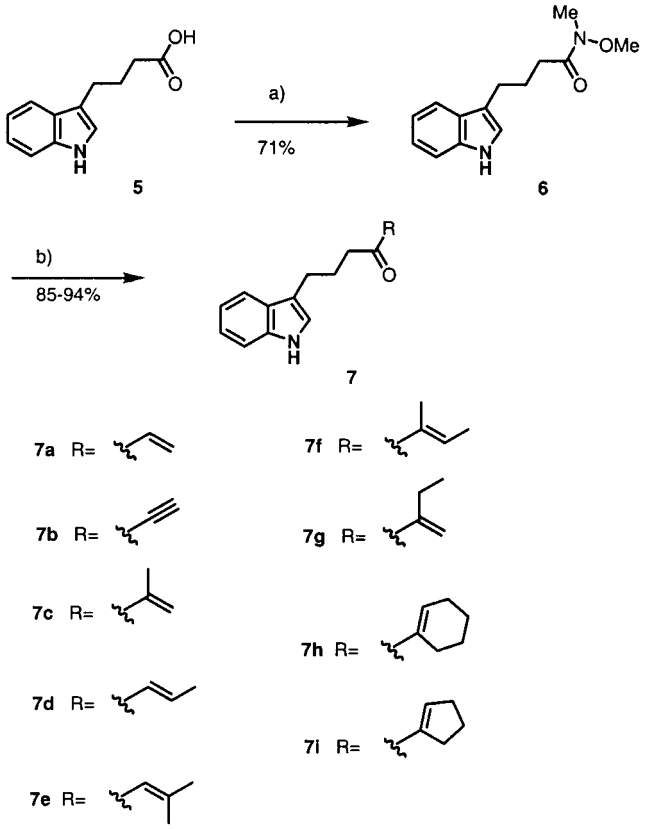
Scheme 2. Synthetic Approach to Enones Described in This Work[a]



[a] Reagents and conditions: (a) HN(Me)OMe-HCl, EDC, DMAP, CH_2Cl_2, rt; (b) Grignard reagent, THF, rt.

was shown to be a very potent inhibitor for AANAT, and the structural basis for inhibition is discussed.

Design and Chemistry

It was our intent to examine three features of the bisubstrate analogue tethering moiety and their contributions to inhibition. First, we wanted to examine the impact of the amide nitrogen as a hydrogen bond donor. The X-ray structure revealed that this NH interacts with a protein backbone carbonyl oxygen atom.[5,6] Second, we hoped to assess indirectly the preference for AANAT binding to a tetrahedral intermediate[8] in the catalytic reaction by preparing an sp^3 carbinol analogue. Third, we were interested in analyzing the relative contributions of added hydrophobicity versus conformational restraint in AANAT inhibition. The set of target compounds prepared in this study are **8a**−**j** and **9**.

The compounds **7a**−**i** were prepared from a common intermediate dimethylhydroxamic acid **6** via Grignard additions[9] as shown in Scheme 2. In most cases, the corresponding vinyl or acetylenic bromides to generate the Grignard reagents were commercially available, although the cyclopentenyl and cyclohexenyl bromides required synthetic preparation following known routes.[10] Michael addition of CoASH to the α,β-unsaturated ketones **7a**−**i** generated analogues **8a**−**j** (Scheme 3). Of note, addition of CoASH to a number of the conjugated ketones led to diastereomeric centers (at the α and/or β carbons relative to the carbonyls of compounds **8c**, **8d**, and **8f**−**8j**) which proved impossible to resolve by reversed-phase HPLC. In most cases these Michael additions were relatively rapid at room temperature, although addition to the cyclohexene system required warming to 55 °C and extended reaction time. The diastereomeric carbinols **9** were afforded from **8a** using $NaBH_4$ reduction (Scheme 4) and were not separable by HPLC. While diastereomeric mixtures were present in **8c**, **8d**, **8f**−**8j**, and **9**, it is quite likely that most are random distributions (1:1 for **8c**, **8d**, **8g**−**8i**, **9**) since the nearest chiral center which could influence reactivity is remote from the site of the stereocenter generated in the CoASH addition or $NaBH_4$ reduction steps. The distribution of the two stereoisomers (erythro/threo ratio) generated from CoASH addition to **7f** relating to the vicinal methyls present in the butyl moiety in **8f** cannot, however, be predicted with high confidence. Nevertheless, for all of the other compounds, it can be presumed that the inhibitory concentrations employed in the assays for the most potent diastereomers are within 2-fold of the amount stated for the total concentration of the diastereomeric mixtures.

AANAT Inhibition

Compounds **8a**−**j** and **9** were evaluated as potential inhibitors of AANAT using the previously developed spectrophotometric assay.[2] The values given are apparent K_is taken from Dixon kinetic analyses with the assumption that these compounds obeyed a model of linear competitive inhibition against acetyl-CoA and noncompetitive inhibition against tryptamine.[4]

Inhibition by 8a and 9

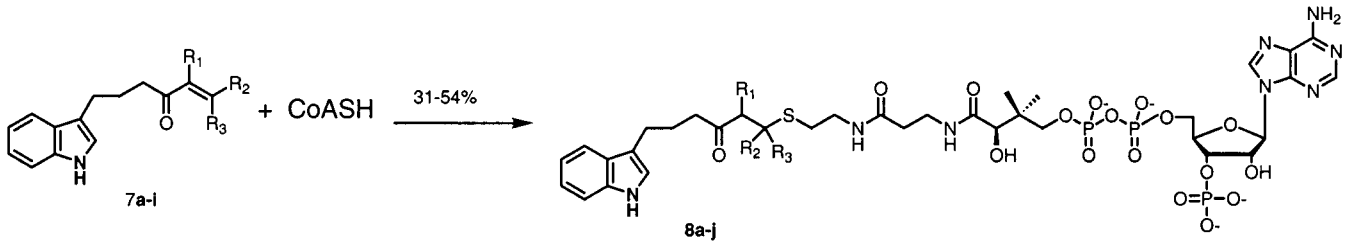
Compound **8a** (Table 1) is the deaza analogue of **3**, a compound synthesized and studied previously.[4] It was shown to have an apparent K_i about 2-fold lower than that previously measured for **3**, and 1.5-fold lower than that of the parent compound **2**.[4] This was an important result as it showed that the amide nitrogen of **2** was dispensable for a high affinity interaction with AANAT. It appears that the hydrogen bond observed in the X-ray structure of AANAT bound to **2** in which the N−H of **2** interacts with a protein backbone carbonyl[5,6] does not contribute significantly to the overall binding energy of **2** in its complex with AANAT. Another possibility is that the loss of the hydrogen bond donating capability of **8a** is offset by a greater tendency to form a tetrahedral intermediate analogue as a hydrate. If this were true, the carbinol **9** might be an even better AANAT inhibitor than **8a**.

The diastereomeric carbinol mixture **9** proved to be considerably weaker as an AANAT inhibitor than the ketone (Table 1). Such carbinols like the classical compound pepstatin[8] are often potent acid protease inhibitors by virtue of mimicking tetrahedral intermediates formed on these enzymes. The X-ray structure of the AANAT complexed with **2** shows no obvious oxyanion hole. These results are consistent with a family of related tetrahedral mimics for other acetyltransferase enzymes, wherein binding of the analogues was weak.[11]

Conformationally Constrained Analogues

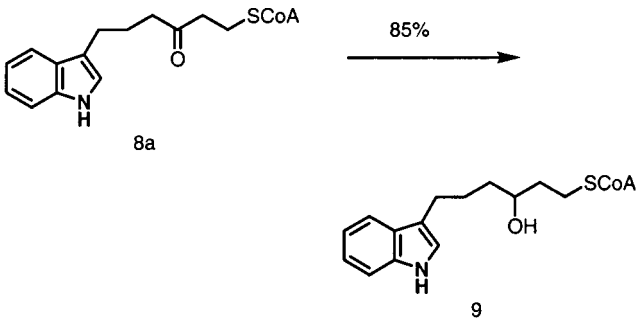
The findings above encouraged a further exploration of deaza bisubstrate ketone analogues as AANAT inhibitors. Given the previously reported findings that substitution of the methylene connector with a methyl

Scheme 3. Conjugate Addition of CoASH to Enones[a]



[a] Reagents and conditions: MeOH/1 M triethyl ammonium bicarbonate aqueous buffer (pH 8.1), rt.

Scheme 4. Reduction of Compound **8a** with $NaBH_4$[a]



[a] Reagents and conditions: $NaBH_4$, MeOH, rt.

group as present in **4** increased the relative potency compared to **2**,[4] we investigated a related analogue **8c** (Table 1). Compound **8c** (K_i = 6 nM) was shown to have 5-fold enhanced potency with respect to **8a** (K_i = 32 nM). This established that the bisubstrate ketone analogues as exemplified by **8** and the amide analogues such as **2** likely show similar binding modes to AANAT. To further probe conformational preferences within the AANAT active site, the trans and cis olefins **8be** and **8bz** were tested (Table 1). These rigidly structured tethered molecules were both weak AANAT inhibitors, blocking the enzyme approximately 1000−2000 times more weakly compared to **8a**. It is deduced that the locked conformations of these compounds do not correspond to the optimal binding mode of **8a** or **8c**.

To further analyze the bisubstrate analogue tether requirements, another set of analogues containing five- or six-membered rings was screened. The cyclohexyl compound **8h** having the ring substituents with the cis configuration proved to be a modest AANAT inhibitor with apparent K_i of 942 nM (Table 1). As the cyclohexyl trans isomer did not appear to be generated to a significant extent in the CoASH coupling reaction, its inhibitory potency was not measured. Interestingly, both cyclopentyl stereochemical isomers **8i** and **8j** with apparent K_i's of 65 nM and 7 nM, respectively (Table 1), proved to be more potent inhibitors than **8h**. Distinguishing which cyclopentyl ring isomer was cis versus trans proved to be impossible by NMR. However, modeling studies based on the AANAT crystal structure suggest that the trans isomer may be more potent.

To further understand the relative potency of **8c** and **8j**, a series of noncyclized, alkyl substituted analogues **8d**−**8g** were evaluated as AANAT inhibitors (Table 1). Compound **8d** showed an apparent K_i of 52 nM, suggesting simple methyl substitution geminal to the sulfur did not enhance binding relative to the parent compound **8a**. Moreover, the dimethyl substituted compounds **8e** and **8f** and the ethyl substituted analogue **8g** were all less potent than **8j**, suggesting that bulkier substitution per se was not responsible for the tight inhibition by **8j**. These studies set a geometric constraint on the conformations necessary for optimal inhibition of AANAT.

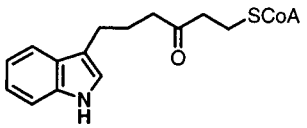
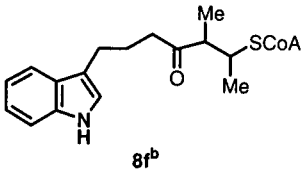
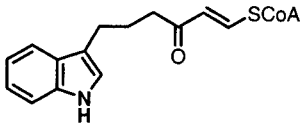
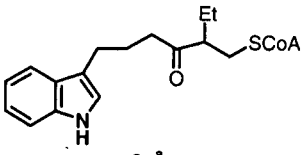
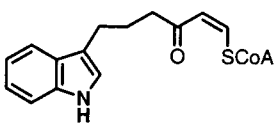
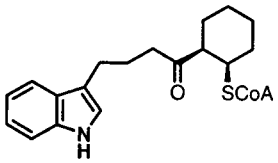
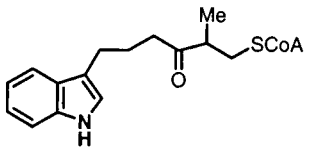
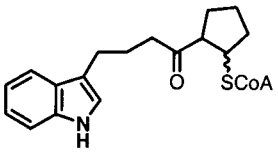
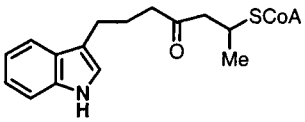
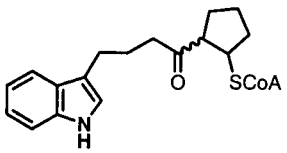
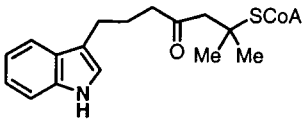
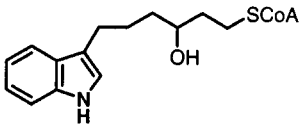
Discussion

The concept that bisubstrate analogues can be potent enzyme inhibitors has been established for 30 years.[12,13] However, the conformational orientation between the two substrates as set by the linking group(s) and the effects of this relationship on inhibitory potency and potentially enzyme mechanism have been somewhat underexplored. Bisubstrate analogues may be potent inhibitors because of their mimicry of the transition state. Alternatively, bisubstrate analogues in which orientation of the two substrates simulates the ground-state 'near-attack conformation' (NAC) may show optimal enzyme inhibition.[14,15]

In an effort to determine structural features that are most critical for AANAT blockade as well as to generate concepts applicable to understanding enzyme mechanism, a series of bisubstrate ketone analogues were synthesized and explored as AANAT inhibitiors. Among the salient findings were the apparent unimportance of the bisubstrate analogue bridging nitrogen in **2**, as illustrated by the potent inhibition by the ketone **8a**, and the lack of tetrahedral stabilization likely required in the acetyl transfer mechanism as exemplified by the weak inhibition by the carbinol **9**. This latter finding may have general relevance to the aminolysis of reactive esters catalyzed by enzymes. In contrast to the difficult cleavage of amides, the chemical ease of acyl transfer of an activated ester to an amine is well-established.[16] Thus enzymes that catalyze this reaction might derive little benefit by stabilizing a tetrahedral intermediate using, for example, an 'oxyanion hole'. Rather, bringing the two substrates together in an attack conformation and assisting in the deprotonation of the serotonin (tryptamine) ammonium ion to afford the neutral amine may be the primary enzymatic contribution.

Results from the array of conformationally rigidified tethers investigated produce a model for how the two substrates are aligned within the active site. In particular, the inclusion of a methyl group (**8c**) or cyclopentyl framework (**8j**) provide the highest apparent affinity to AANAT. Of these two compounds, the cyclopentyl compound has the most rotational constraints and thus should ultimately provide the greatest insight into AANAT recognition. Results with the acyclic tethers show that increasing the alkyl substitution (and the hydrophobic content) per se did not enhance inhibition compared to **8a** and **8c**, supporting the concept that conformtional restraint is key. Of note, several of the

Table 1. K_i Values of AANAT Bisubstrate Inhibitors Measured in This Work

Compound	K_i (nM)	Compound	K_i (nM)
 **8a**	32	 **8f**[b]	59
 **8be**	39,900	 **8g**[a]	21
 **8bz**	71,200	 **8h**[a]	942
 **8c**[a]	6	 **8i**[a,c]	65
 **8d**[a]	52	 **8j**[a,c]	7
 **8e**	275	 **9**[a]	1,100

[a] This K_i was based on the diastereomeric mixture of two compounds. [b] This K_i was based on a diastereomeric mixture of up to four compounds. [c] The relative stereochemistry for this ring isomer is undetermined. Standard error on these values is ±20%.

compounds tested consisted of diastereomeric mixtures which proved impossible to separate by HPLC. This limits the ability of modeling the optimized geometry for inhibition. Attempts to obtain the X-ray structure of AANAT complexed with **8j** are underway, and this should ultimately reveal the preferred conformation for inhibition.

While these studies have focused on only one acetyltransferase, it is likely that they will have relevance to other members of the GNAT superfamily. Recently published studies suggest that PCAF, a histone acetyltransferase member, is also potently inhibited by bisubstrate analogues connected by an acetyl linker.[17] In unpublished work, it has been shown that a peptide analogue of **4** (methyl group-containing) was more potent than the unsubstituted version (CMK and PAC, unpublished data). It is therefore plausible that related, conformationally restrained ketone tethers can be used to achieve greater potency for various GNAT acetyltransferases.

An area not addressed in the current studies concerns inhibitor bioavailability. The negatively charged AANAT inhibitors described here are unlikely to be cell permeable.[18] To overcome this obstacle, pro-drug variants of **8c** and **8j** can be considered. In situ generation of compound **2** was shown to be possible by nucleophilic reaction of the requisite bromo-acetamide with CoASH in an AANAT catalyzed fashion.[4] Such an approach may also be possible for **8c** and **8j**.

Conclusions

We have described a new series of bisubstrate ketone analogues and discovered two particularly potent AANAT inhibitors. A tetrahedral intermediate bisubstrate mimic

only weakly blocked AANAT action. The relative orientation of the two substrate moieties with respect to each other appears to be important for potent inhibition and can be optimized with conformationally restrained linkers. Future studies will involve improving the bioavailability of these compounds and determining their structural basis for inhibition.

Experimental Section

General. All reactions with air or moisture sensitive reagents and solvents were carried out under a nitrogen atmosphere. In general, reagents and solvents were used as purchased without further purification. All reagents except coenzyme A were purchased from Aldrich. Coenzyme A (approximately 95%, Li salt) was purchased from Sigma. Column chromatography was performed with silica gel (70−230 mesh, Merck). Analytical TLC was performed on Baker-Flex TLC plate (silica gel IB-F), and the plates were visualized with a Spectroline UV lamp (at 254 nm). Target molecules containing coenzyme A were separated by reversed-phase C-18 preparatory HPLC (MICROSORBTM-100, RAININ) with methanol and 0.05 M potassium phosphate solution (pH 4.5). The progress of reactions of coenzyme A with alkylating reagent and the purity of the target compounds were monitored by reversed-phase C-18 analytical HPLC (MICROSORB-MVTM-100, RAININ; (column void volume = 3 mL)). The detection of compounds was performed with the UV lamp wavelength set at 260 nm. The purities of all target molecules were more than 95% (excluding consideration of diastereomeric mixtures) based on analytical HPLC results in two solvent systems. Proton NMR spectra were recorded on a Bruker AX-300 (300 MHz) or Varian VI series (600, 500, or 400 MHz) instruments; chemical shifts of signals are expressed in parts per million (ppm) and are referenced to the deuterated solvents used. Carbon-13 NMR spectra were recorded on a Varian VI series (100 or 125 MHz) instruments; chemical shifts are expressed in ppm and are referenced to the deuterated solvents used. One-dimensional homo decoupling, two-dimensional proton−proton COSY, and two-dimensional NOESY spectra were recorded on a Varian VI 600 (600 MHz). Coupling constants (*J*) are given in hertz. High-resolution mass spectra were recorded on VG-ZAB or DE-STR. The preparation of recombinant sheep glutathione S-transferase(GST)-AANAT (∼90% pure), which was expressed in *Escherichia coli* has been described previously,[2] and it has also been shown that nearly identical kinetic behavior was observed for GST-AANAT and GST-free AANAT.[2] Therefore, GST-AANAT was used for all the studies described in this manuscript.

3-((4′-*N*,*O*-Dimethyl hydroxyamido)-butan)-indole (6). To a stirred solution of 3-indole butyric acid **5** (2.1 g, 10.3 mmol), *N*,*O*-dimethyl hydroxy amine hydrochloride (1.1 g, 11.4 mmol), and 4-(dimethylamino)-pyridine (1.4 g, 11.4 mmol) in dichloromethane (100 mL) was portionwise added 1-(3-dimethylamino propyl) 3-ethylcarbodiimide hydrochloride (2.2 g, 11.4 mmol) for 2 h. After an additional 4 h at room temperature, the reaction mixture was quenched with water (100 mL) and then extracted with ethyl acetate (30 mL × 3). The extract was dried over magnesium sulfate and concentrated in vacuo. The residue was purified by column chromatography (AcOEt: hexane = 1:1) affording 1.71 g of **6** (61%) and 0.63 g of recovered **5** (30%). ^{1}H NMR (300 MHz, $CDCl_3$): 2.11 (m, 2H); 2.55 (m, 2H); 2.87 (m, 2H); 3.22 (s, 3H); 3.63 (s, 3H); 7.00 (s, 1H); 7.18 (m, 2H); 7.37 (d, J = 7.8 Hz, 1H); 7.65 (d, J = 7.8 Hz, 1H); 8.24 (bs, NH, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): 24.66; 24.88; 31.50; 61.07; 61.13; 111.02; 115.87; 118.90; 119.01; 121.37; 121.77; 127.49; 136.32; 174.68. HRMS (M^+) calcd for $C_{14}H_{18}N_2O_2$ 246.1368, found 246.1366.

Typical Procedure for Formation of Conjugated Ketone. To an ice cooled stirred suspension of **6** (0.5 mmol) in tetrahydrofuran (2 mL) was added alkenylmagnesium bromide (1.5 mmol). After being left overnight at room temperature, the reaction mixture was quenched with saturated ammonium chloride (3 mL) and then extracted with diethyl ether (3 mL × 4). After drying over magnesium sulfate the residue was concentrated in vacuo. Column chromatography (EtOAc:hexane = 1:5) of crude product gave correspondent conjugated ketones.

3-(Hex-5′-en-4′-on)-indole (7a). The compound was prepared from ethenylmagnesium bromide as described, yielding 94% of **7a**. ^{1}H NMR (300 MHz, $CDCl_3$): 2.05 (m, 2H); 2.65 (m, 2H); 2.80 (m, 2H); 5.77 (dd, J = 10.5 and 1.2 Hz, 1H); 6.16 (dd, J = 17.7 and 1.2 Hz, 1H); 6.33 (dd, J = 10.5 and 17.7 Hz, 1H); 6.98 (s, 1H); 7.15 (m, 2H); 7.35 (d, J = 7.8 Hz, 1H); 7.60 (d, J = 7.8 Hz, 1H); 7.96 (bs, 1H, NH) ^{13}C NMR (125 MHz, $CDCl_3$): 24.20; 24.37; 39.06; 111.07; 115.50; 118.76; 119.00; 121.45; 121.74; 127.34; 127.98; 136.28; 136.42; 201.13. HRMS (M^+) calcd for $C_{14}H_{15}N_1O_1$ 213.1149, found 213.1154.

3-(Hex-5′-yn-4′-on)-indole (7b). The compound was prepared from ethynylmagnesium bromide as described, yielding 89% of **7b**. ^{1}H NMR (400 MHz, $CDCl_3$): 2.13 (m, 2H); 2.67 (m, 2H); 2.83 (m, 2H); 3.21 (s, 1H); 6.96 (s, 1H); 7.15 (m, 1H); 7.25 (m, 1H); 7.36 (d, J = 8.0 Hz, 1H); 7.63 (d, J = 8.0 Hz, 1H); 8.10 (bs, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): 24.03; 24.10; 44.87; 78.56; 81.33; 111.11; 115.03; 118.70; 119.09; 121.58; 121.83; 127.22; 136.27; 187.57. HRMS (M^+) calcd for $C_{14}H_{18}N_1O_1$ 211.0997, found 211.1005.

3-(5′-Methyl-hex-5′-en-4′-on)-indole (7c). The compound was prepared from isopropenylmagnesium bromide as described, yielding 94% of **7c**. ^{1}H NMR (400 MHz, $CDCl_3$): 1.97 (s, 3H); 2.14 (m, 2H); 2.82 (m, 2H); 2.87 (m, 2H); 5.78 (s, 1H); 5.95 (s, 1H); 6.95 (s, 1H); 7.27 (m, 2H); 7.37 (d, J = 7.8 Hz, 1H); 7.71 (d, J = 7.8 Hz, 1H); 8.38 (bs, 1H, NH). ^{13}C NMR (100 MHz, $CDCl_3$): 17.43; 24.35; 24.70; 36.81; 111.05; 115.25; 118.59; 118.76; 121.45; 121.49; 124.66; 127.21; 136.18; 144.03; 202.53. HRMS (M^+) calcd for $C_{15}H_{17}N_1O_1$ 227.1310, found 227.1306.

3-(Hept-5′-en-4′-on)-indole (7d). The compound was prepared from 1-propenylmagnesium bromide as described, yielding 85% of **7d**. ^{1}H NMR (400 MHz, $CDCl_3$): 1.86 (dd, J = 6.8 and 1.6 Hz, 3H); 2.07 (m, 2H); 2.61 (m, 2H); 2.81 (m, 2H); 6.11 (dq, J = 16 and 1.6 Hz, 1H); 6.81 (m, 1H); 6.95 (s, 1H); 7.16 (m, 2H); 7.35 (d, J = 7.8hz, 1H); 7.62 (d, J = 7.8 Hz, 1H); 8.17 (bs, 1H, NH). ^{13}C NMR (100 MHz, $CDCl_3$): 18.10; 24.42; 24.46; 39.39; 111.01; 115.62; 118.77; 118.96; 121.40; 121.71; 127.35; 131.80; 136.25; 142.51; 200.76. HRMS (M^+) calcd for $C_{15}H_{17}N_1O_1$ 227.1310, found 227.1306.

3-(6′-Methyl-hept-5′-en-4′-on)-indole (7e). The compound was prepared from 2-methyl-2-propenylmagnesium bromide as described, yielding 92% of **7e**. ^{1}H NMR (400 MHz, $CDCl_3$): 1.90 (s, 3H); 2.10 (m, 2H); 2.22 (s, 3H); 2.55 (m, 2H); 2.84 (t, 2H); 6.10 (s, 1H); 6.93 (s, 1H); 7.21 (m, 2H); 7.35 (d, J = 7.8 Hz, 1H); 7.67 (d, J = 7.8 Hz, 1H); 8.52 (bs, 1H, NH). ^{13}C NMR (100 MHz, $CDCl_3$): 20.51; 24.35; 24.49; 27.38; 43.67; 111.02; 115.30; 118.58; 118.68; 121.41; 121.46; 123.65; 127.24; 136.20; 154.85; 201.44. HRMS (M^+) calcd for $C_{16}H_{19}N_1O_1$ 241.1467, found 241.1467.

3-(5′-Methyl-hept-5′-en-4′-on)-indole (7f). The compound was prepared from 1-methyl-1-propenylmagnesium bromide as described, yielding 92% of **7f** (inseparable 1:1 mixture of *Z* and *E* based on proton NMR). ^{1}H NMR (400 MHz, $CDCl_3$, integration for two compounds): 1.86 (m, 12H); 2.08 (m, 4H); 2.63 (m, 2H); 2.75 (m, 2H); 2.82 (m, 4H); 5.80 (m, vinyl-H, 1H); 6.79 (m, vinyl-H, 1H); 6.94 (s, 2H); 7.14 (m, 4H); 7.34 (d, J = 8.0 Hz, 2H); 7.65 (d, J = 8.0 Hz, 2H); 8.26 (s, 2H, NH). ^{13}C NMR (100 MHz, $CDCl_3$): 10.93; 14.60; 15.53; 20.61; 24.05; 24.44; 24.54; 25.14; 36.62; 41.43; 111.05; 115.57; 115.63; 118.71; 118.74; 118.90; 121.41; 121.64; 127.32; 132.40; 136.26; 136.28; 137.28; 138.00; 202.20; 206.11. HRMS (M^+) calcd for $C_{16}H_{19}N_1O_1$ 241.1467, found 241.1461.

3-(5′-Ethyl-hex-5′-en-4′-on)-indole (7g). The compound was prepared from 2-butenylmagnesium bromide as described, yielding 86% of **7g**. ^{1}H NMR (400 MHz, $CDCl_3$): 1.00 (m, 3H); 2.03 (m, 2H); 2.27 (m, 2H); 2.76 (m, 4H); 5.65 (bs, 1H); 5.90 (s, 1H); 6.97 (bs, 1H); 7.13 (m, 2H); 7.33 (d, J = 8.0 Hz, 1H); 7.59 (d, J = 8.0 Hz, 1H); 7.94 (bs, 1H, NH). ^{13}C NMR (100 MHz, $CDCl_3$): 12.58; 23.68; 24.51; 24.78; 37.34; 110.97; 115.98;

118.90; 119.11; 121.27; 121.86; 122.56; 127.42; 136.27; 150.27; 202.17. HRMS (M^+) calcd for $C_{16}H_{19}N_1O_1$ 241.1467, found 241.1470.

3-(4′-Cyclohexenyl butan-4′on)-indole (7h). The compound was prepared from cyclohexenylmagnesium bromide as described, yielding 91% of **7h**. ^{1}H NMR (300 MHz, $CDCl_3$): 1.64 (bm, 4H); 2.07 (m, 2H); 2.24 (bm, 4H); 2.74 (m, 2H); 2.83 (m, 2H); 6.85 (s, vinyl-H, 1H); 7.06 (d, J = 8.1 Hz, 1H); 7.21 (m, 2H); 7.37 (d, J = 8.1 Hz, 1H); 7.64 (d, J = 7.5 Hz, 1H); 8.12 (bs, NH, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): 21.53; 21.94; 23.11; 24.64; 25.06; 25.98; 36.56; 111.02; 116.00; 118.91; 119.05; 121.36; 121.80; 127.48; 136.31; 139.11; 139.70; 201.73. HRMS (M^+) calcd for $C_{18}H_{21}N_1O_1$ 267.1623, found 267.1617.

3-(4′-Cyclopentenyl butan-4′on)-indole (7i). The compound was prepared from cyclopentenylmagnesium bromide as described yielded 91% of **7i**. ^{1}H NMR (300 MHz, $CDCl_3$): 1.95 (m, 2H); 2.10 (m, 2H); 2.57 (m, 4H); 2.77 (m, 2H); 6.68 (d, vinyl-H, J = 1.8 Hz, 1H); 6.99 (s, 1H); 7.18 (m, 2H); 7.37 (d, J = 8.1 Hz, 1H); 7.65 (d, J = 8.1 Hz, 1H); 8.27 (bs, NH, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): 22.56; 24.47; 24.86; 30.49; 33.72; 38.39; 111.05; 115.40; 118.64; 118.77; 121.47; 121.51; 127.29; 136.23; 143.58; 145.28; 199.61. HRMS (M^+) calcd for $C_{17}H_{19}N_1O_1$ 253.1467, found 253.1461.

Typical Procedure for Formation of Bisubstrate Analogue 8. The conjugated ketone (2−3 equiv) was dissolved in MeOH [33 mL/mmol of coenzyme A with free sulfhydryl group (CoASH)] that was freshly degassed by bubbling N_2. To that solution was added a solution of CoASH (1 equiv) in a buffered solution of 1 M triethylammonium bicarbonate buffer (33 mL/mmol, pH 7.9∼8.3). The mixture was stirred under N_2. Reaction progress was monitored by using 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) and analytical HPLC, and the reaction was generally complete within 1 h at room temperature, except for conjugated ketone **7h** which was heated at 55 °C for 16 h. The reaction mixture was concentrated in vacuo to remove MeOH. The remaining aqueous solution was diluted with H_2O to ∼5 mL and was extracted with EtOAc (3 × 5 mL) to remove excess alkylating reagent. The remaining solution was purified on a reversed-phase C-18 HPLC column by using MeOH/KH_2PO_4 (5 0mM, pH ∼4.5) gradient elution. The compound was desalted by reinjection on the same HPLC column, washing with H_2O for ∼25 min, then eluting the pure compound with 100% MeOH. Compounds were lyophilized to dryness and were quantified by weight and UV (absorption at 279 nm on the basis of the extinction coefficient for acetyl-CoA and tryptamine in H_2O).

Typical Preparatory HPLC Gradient Method. Flow rate = 10 mL/min; 100% A (0.05M KH_2PO_4) for 5 min, followed by a linear gradient to 65% B (MeOH) over 50 min and then to 80% B over the next 15 min. In this gradient, the retention time was generally 52−60 min.

Analytical HPLC. After purification, all bisubstrate analogues **8** and **9** appeared homogeneous (>95% pure) in two separate reversed-phase (C-18) HPLC systems with UV monitoring at 260 nm. The first analytical HPLC gradient method to analyze purity employed 0.05 M KH_2PO_4 (A)/ MeOH (B) solution (pH 4−5); flow rate = 1 mL/min; 100%A for 3 min followed by a linear gradient to 65%B over 15 min, then a linear gradient to 100%B over the next 5 min, then 100%B until completion. In this gradient, the retention time for compounds **8** and **9** was in the range of 17−21 min. In the second system, the solvents used were water−0.05% trifluoroacetic acid (A)/ CH_3CN−0.05% trifluoroacetic acid (B); the flow rate = 1 mL/min; starting from 40%B, there was a linear gradient to 100% B over 15 min. In this system, compounds **8** and **9** showed retention times in the range of 5−7 min.

Bisubstrate Analogue 8a. The compound was prepared from conjugated ketone **7a** as described, yielding 31% of **8a**. ^{1}H NMR (300 MHz, D_2O): 0.59 (s, 3H); 0.73 (s, 3H); 1.79 (m, 2H); 2.27 (m, 2H); 2.39 (m, 6H); 2.50 (m, 2H); 2.58 (m, 2H); 3.12 (m, 2H); 3.29 (bm, 2H); 3.41 (m, 1H); 3.70 (m, 1H); 3.87 (s, 1H); 4.10 (bs, ribose 5′-CH_2, 2H); 4.44 (m, ribose H, 1H); 4.68 (s, ribose H, 2H); 5.98 (m, ribose anomeric H, 1H); 7.03 (m, 3H); 7.28 (d, J = 8.1 Hz, 1H); 7.44 (d, J = 8.4 Hz, 1H); 8.01 (s, adenine 2-H, 1H); 8.35 (s, adenine 8-H, 1H). HRMS ($M + H^+$) calcd for $C_{35}H_{52}N_8O_{17}P_3S$ 981.2379, found 981.2395.

Bisubstrate Analogue 8be and 8bz. A mixture was prepared from conjugated ketone **7b** as described**,** yielding 32% of **8be** and 5% of **8bz**. **8be**: ^{1}H NMR (300 MHz, CD_3OD): 0.83 (s, 3H); 1.05 (s,3H); 2.00 (m, 2H); 2.42 (m, 2H); 2.57 (m, 2H); 2.76 (m, 2H); 2.92 (m, 2H); 3.37 (m, 2H); 3.47 (m, 2H); 3.59 (m, 1H); 3.98 (m, 1H); 4.06 (s, 1H); 4.26 (bs, ribose 5′-CH_2, 2H); 4.49 (s, ribose H, 1H); 6.11 (d, J = 6.0 Hz, ribose anomeric H, 1H); 6.25 (d, J = 15.3 Hz, vinyl-H, 1H); 7.02 (m, 3H); 7.31 (d, J = 7.8 Hz, 1H); 7.5 (d, J = 7.8 Hz, 1H); 7.65 (d, J = 15.6 Hz, vinyl-H, 1H); 8.18 (s, adenine 2-H, 1H); 8.55 (s, adenine 8-H, 1H). HRMS ($M + H^+$) calcd for $C_{35}H_{50}N_8O_{17}P_3S$ 979.2222, found 979.2237. The *E* configuration is assigned based on the larger (15 Hz) vinyl proton coupling constant. **8bz**: ^{1}H NMR (300 MHz, D_2O): 0.53 (s, 3H); 0.72 (s,3H); 1.93 (m, 2H); 2.39 (m, 2H); 2.49 (m, 2H); 2.64 (m, 2H); 2.84 (m, 2H); 3.38 (bm, 4H); 3.53 (m, 1H); 3.82 (m, 1H); 3.97 (s, 1H); 4.21 (bs, ribose 5′-CH2, 2H); 4.55 (m, ribose H, 1H); 6.06 (m, ribose anomeric H, 1H); 6.34 (d, J = 9.0 Hz, vinyl-H, 1H); 7.01 (s, 1H); 7.13 (m, 2H); 7.18 (d, J = 9.9 Hz, vinyl-H, 1H); 7.34 (d, J = 7.8 Hz, 1H); 7.48 (d, J = 7.8 Hz, 1H); 8.11 (s, adenine 2-H, 1H); 8.51 (s, adenine 8-H, 1H). HRMS ($M + H^+$) calcd for $C_{35}H_{50}N_8O_{17}P_3S$ 979.2222, found 979.2242. The *Z* configuration is assigned based on the smaller (9 Hz) vinyl proton coupling constant.

Bisubstrate Analogue 8c. The compound was prepared from conjugated ketone **7c** as described, yielding 54% of **8c**. ^{1}H NMR (300 MHz, D_2O): 0.76 (s, 3H); 0.90 (s, 3H); 1.04 (d, J = 6.9 Hz, 3H); 1.95 (m, 2H); 2.42 (m, 2H); 2.53 (m, 3H); 2.61 (m, 3H); 2.73 (m, 2H); 2.82 (m, methinyl, 1H); 3.22 (m, 2H); 3.44 (bm, 2H); 3.58 (m, 1H); 3.85 (m, 1H); 4.04 (s, 1H); 4.27 (bs, ribose 5′-CH_2, 2H); 4.61 (m, ribose H, 1H); 4.70 (s, ribose H, 2H); 6.13 (d, J = 5.7 Hz, ribose anomeric H, 1H); 7.16 (m, 3H); 7.43 (d, J = 8.1 Hz, 1H); 7.60 (d, J = 8.4 Hz, 1H); 8.18 (s, adenine 2-H, 1H); 8.52 (s, adenine 8-H, 1H). HRMS ($M - 3H^+ + 4K^+$) calcd for $C_{36}H_{50}N_8O_{17}P_3SK_4$ 1147.0770, found 1147.0750.

Bisubstrate Analogue 8d. The compound was prepared from conjugated ketone **7d** as described, yielding 38% of **8d**. ^{1}H NMR (400 MHz, CD_3OD): 0.72 (s, 3H); 0.98 (s, 3H); 1.11 (d, J = 6.8 Hz, 3H); 1.86 (m, 2H); 2.33 (m, 2H); 2.40 (m, 4H); 2.50 (m, 2H); 2.57 (m, 1H); 2.65 (m, 2H); 3.09 (m, 2H); 3.19 (m, 2H); 3.47 (m, 1H); 3.93 (m, 1H); 3.99 (s, 1H); 4.18 (s, ribose 5′-CH_2, 2H); 4.39 (m, ribose H, 1H); 4.68 (s, ribose H, 2H); 6.03 (d, J = 6.0 Hz, ribose anomeric H, 1H); 6.91 (m, 3H); 7.22 (d, J = 8.1 Hz, 1H); 7.41 (d, J = 8.4 Hz, 1H); 8.10 (s, adenine 2-H, 1H); 8.49 (s, adenine 8-H, 1H). HRMS ($M - 3H^+ + 4K^+$) calcd for $C_{36}H_{50}N_8O_{17}P_3SK_4$ 1147.0770, found 1147.0840.

Bisubstrate Analogue 8e. The compound was prepared from conjugated ketone **7e** as described, yielding 32% of **8e**. ^{1}H NMR (300 MHz, CD_3OD): 0.83 (s, 3H); 1.07 (s,3H); 1.33 (s, 6H); 1.92 (m, 2H); 2.41 (m, 2H); 2.52 (m, 2H); 2.59 (m, 2H); 2.63 (s, 2H); 2.74 (m, 2H); 3.23 (m, 2H); 3.46 (bm, 2H); 3.59 (m, 1H); 4.00 (m, 1H); 4.08 (s, 1H); 4.28 (bm, ribose 5′-CH_2, 2H); 4.50 (bm, ribose H, 1H); 4.89 (m, ribose H, 2H); 6.12 (d, J = 6.4 Hz, ribose anomeric H, 1H); 7.03 (m, 3H); 7.30 (d, J = 8.1 Hz, 1H); 7.50 (d, J = 8.4 Hz, 1H); 8.19 (s, adenine 2-H, 1H); 8.57 (s, adenine 8-H, 1H). HRMS ($M - 3H^+ + 4K^+$) calcd for $C_{36}H_{50}N_8O_{17}P_3SK_4$ 1161.0927, found 1161.0930.

Bisubstrate Analogue 8f. The mixture was prepared from conjugated ketone **7f** as described, yielding 49% of **8f** (inseparable mixture of several diastereomers). ^{1}H NMR (300 MHz, CD_3OD, integration for two compounds): 0.84 (m, 6H); 1.07 (m, 6H); 1.09 (d, J = 6.9 Hz, 6H); 1.22 (d, J = 6.6 Hz, 6H); 1.94 (m, 4H); 2.42 (m, 4H); 2.59 (m, 10H); 2.75 (m, 4H); 3.02 (m, 2H); 3.27 (m, 4H); 3.46 (m, 4H); 3.62 (bm, 2H); 4.02 (m, 2H); 4.06 (s, 2H); 4.33 (bs, ribose 5′-CH_2, 4H); 4.50 (bs, ribose H, 2H); 4.77 (m, ribose H, 4H); 6.10 (d, J = 3.0 Hz, ribose anomeric H, 2H); 7.00 (m, 6H); 7.31(d, J = 6.0 Hz, 2H); 7.51 (d, J = 9.0 Hz, 2H); 8.21 (s, adenine 2-H, 2H); 8.64 (s, adenine 8-H, 2H). HRMS ($M - 3H^+ + 4K^+$) calcd for $C_{36}H_{50}N_8O_{17}P_3SK_4$ 1161.0927, found 1161.0936.

Bisubstrate Analogue 8g. The mixture was prepared from conjugated ketone **7g** as described, yielding 51% of **8g**. ^{1}H NMR (400 MHz, CD_3OD): 0.72 (m, ethyl CH_3, 3H); 0.79 (s,

3H); 0.96 (s, 3H); 1.45 (m, ethyl CH_2, 2H); 1.85 (m, 2H); 2.30 (m, 2H); 2.45 (m, 5H); 2.56 (m, 2H); 2.65 (m, 2H); 3.16 (m, 2H); 3.35 m, 2H); 3.46 (m, 1H); 3.91 (m, 1H); 3.98 (s, 1H); 4.17 (bt, ribose 5′-CH_2, 2H); 4.39 (bs, ribose H, 1H); 4.71 (s, ribose H, 2H); 6.02 (d, J = 6.0 Hz, ribose anomeric H, 1H); 6.91 (m, 3H); 7.20 (d, J = 8.8 Hz, 1H); 7.41 (d, J = 8.0 Hz, 1H); 8.09 (s, adenine 2-H, 1H); 8.48 (s, adenine 8-H, 1H). HRMS ($M - 3H^+ + 4K^+$) calcd for $C_{37}H_{52}N_8O_{17}P_3SK_4$ 1161.0927, found 1161.0977.

Bisubstrate Analogue 8h. The mixture was prepared from conjugated ketone **7h** as described, yielding 43% of **8h**. ^{1}H NMR (600 MHz, D_2O): 0.72 (s, 3H); 0.87 (s, 3H); 1.11 (m, 1H); 1.33 (m, 1H); 1.37 (m, 1H); 1.40 (m, 1H); 1.46 (m, 2H), 1.59 (m, 2H); 1.77 (m, 1H); 1.90 (m, 2H); 2.34 (m, 4H); 2.53 (m, 2H); 2.68 (m, coupling on m at 1.33 and 1.37 ppm, and bs at 3.22 ppm; NOE on bs at 3.2 ppm, axial, 1H); 2.72 (m, 2H); 3.04 (m, 2H); 3.22 (bs, coupling on m at 1.59, 1.77, and 2.68 ppm; NOE on m at 2.34, 2.53 and 2.68 ppm, equatorial, 1H); 3.38 (m, 2H); 3.55 (m, 1H); 3.83 (m, 1H); 4.00 (s, 1H); 4.23 (bs, ribose 5′-CH_2, 2H); 4.57 (s, ribose H, 1H); 4.82 (m, ribose H, 2H); 6.11 (d, J = 6 Hz, ribose anomeric H, 1H); 7.10 (s, 1H); 7.06−7.15 (m, 2H); 7.40 (d, J = 7.8 Hz, 1H); 7.58 (d, J = 7.8 Hz, 1H); 8.16 (bs, adenine 2-H, 1H); 8.48 (s, adenine 8-H, 1H). HRMS ($M + H^+$) calcd for $C_{39}H_{58}N_8O_{17}P_3S$ 1035.2848, found 1035.2892. The cis ring configuration is assigned based on the NOE studies above which indicate the signals at 2.68 and 3.22 ppm correspond to cyclohexyl protons α to the ketone and thio group, respectively.

Bisubstrate analogue 8i and 8j. A mixture was prepared from conjugated ketone **7i** as described, yielding 17% of **8i** and 14% of **8j**. **8i**: ^{1}H NMR (600 MHz, D_2O): 0.73 (s, 3H); 0.87 (s, 3H); 1.56 (m, 1H); 1.65 (m, 2H); 1.73 (m, 2H); 1.88 (m, 2H), 1.96 (m, 1H); 2.35 (m, 2H); 2.39 (m, 2H); 2.50 (m, 1H); 2.57 (m, 1H); 2.70 (m, 2H); 3.09 (m, 2H); 3.20 (m, coupling on m at 1.73 and 3.29 ppm, 1H); 3.29 (m, coupling on m at 1.65, 1.96 and 3.20 ppm, 1H); 3.39 (m, 2H); 3.55 (m, 1H); 3.83 (m, 1H); 4.01 (s, 1H); 4.24 (bs, ribose 5′-CH_2, 2H); 4.58 (s, ribose H, 1H); 4.81 (bm, ribose H, 2H); 6.10 (d, J = 5.4 Hz, ribose anomeric H, 1H); 7.06 (m, 1H); 7.14 (m, 2H); 7.39 (d, J = 7.2 Hz, 1H); 7.55 (d, J = 7.2 Hz, 1H); 8.15 (bs, adenine 2-H, 1H); 8.48 (s, adenine 8-H, 1H). HRMS ($M + H^+$) calcd for $C_{38}H_{56}N_8O_{17}P_3S$ 1021.2692, found 1021.2721. **8j**: ^{1}H NMR (600 MHz, D_2O): 0.72 (bs, 3H); 0.87 (s, 3H); 1.48 (m, 1H); 1.55 (m, 2H); 1.67 (m, 1H); 1.91 (m, 3H); 2.01 (m, 1H); 2.37 (m, 2H); 2.50 (m, 2H); 2.58 (m, 2H); 2.70 (m, 1H); 2.78 (m, coupling on m at 1.55 and 3.14 ppm, 1H); 3.14 (m, coupling on m at 1.48, 1.67 and 2.78 ppm, 1H); 3.19 (m, 2H); 3.40 (m, 2H); 3.54 (m, 1H); 3.83 (m, 1H); 4.00 (s, 1H); 4.23 (bs, ribose 5′-CH_2, 2H); 4.57 (s, ribose H, 1H); 4.81 (bm, ribose H, 2H); 6.10 (d, J = 5.4 Hz, ribose anomeric H, 1H); 7.07 (m, 1H); 7.09 (s, 1H); 7.16 (m, 2H); 7.40 (m, 1H); 7.56 (m, 1H); 8.16 (bs, adenine 2-H, 1H); 8.48 (s, adenine 8-H, 1H) HRMS ($M + H^+$) calcd for $C_{38}H_{56}N_8O_{17}P_3S$ 1021.2692, found 1021.2707.

Bisubstrate Analogue 9. To a stirred solution of **8a** (13 mg, 0.013 mmol) in MeOH (1.5 mL) was added $NaBH_4$ (5 mg, 0.13 mmol) at room temperature. The progress of reaction was checked by analytical reversed-phase HPLC. After 40 min at room temperature the reaction mixture was injected directly to preparatory reverse-phase HPLC using the same solvent system as for the ketones. Desalting followed by lyophilization gave **9** (6 mg) in 46% yield. ^{1}H NMR (300 MHz, D_2O): 0.69 (s, 3H); 0.83 (s, 3H); 1.46 (m, 2H); 1.64 (m, 4H); 2.37 (t, 2H); 2.47 (m, 2H); 2.53 (m, 2H); 2.68 (t, 2H); 3.24 (m, 2H); 3.39 (m, 2H); 3.51 (m, 1H); 3.70 (bm, newly produced H−C−OH, 1H); 3.78 (m, 1H); 3.97 (s, 1H); 4.19 (bs, ribose 5′-CH_2, 2H); 4.53 (s, ribose H, 1H); 4.70 (s, ribose H, 2H); 6.07 (d, J = 5.7 Hz, ribose anomeric H, 1H); 7.07 (s, 1H); 7.10 (m, 2H); 7.38 (d, J = 8.1 Hz, 1H); 7.56 (d, J = 7.5 Hz, 1H); 8.10 (s, adenine 2-H, 1H); 8.45 (s, adenine 8-H, 1H). HRMS ($M + H^+$) calcd for $C_{35}H_{54}N_8O_{17}P_3S$ 983.2535, found 983.2529.

Inhibition of AANAT by Analogues. Assays were carried out in duplicate as previously described with fixed substrate acetyl-CoA (0.3 mM) and tryptamine (0.3 mM) concentrations, and a range of at least five inhibitor concentrations varied around the K_i.[4] Enzyme reactions were initiated with enzyme, quenched at 2 min, and free CoASH levels were determined indirectly by monitoring the reaction products with DTNB at 412 nm. Rate measurements were made under initial conditions, i.e., limiting substrate turnover <10%. All duplicate data agreed within 20% in each run. Kinetics were analyzed by using Dixon plots that were linear in each case. K_i values were estimated assuming a competitive inhibition model vs acetyl-CoA (and noncompetitive vs tryptamine). Although these K_i values are only approximate because of the slow-binding behavior of bisubstrate analogues, previous work shows that there is good agreement between the K_i obtained with this analysis and the K_i* obtained from more extensive time-course measurements described above.[4]

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