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Multivalent binding oligomers inhibit HIV Tat–TAR interaction critical for viral replication

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ABSTRACT

We describe the development of a new type of scaffold to target RNA structures. Multivalent binding oligomers (MBOs) are molecules in which multiple sidechains extend from a polyamine backbone such that favorable RNA binding occurs. We have used this strategy to develop MBO-based inhibitors to prevent the association of a protein–RNA complex, Tat–TAR, that is essential for HIV replication. In vitro binding assays combined with model cell-based assays demonstrate that the optimal MBOs inhibit Tat–TAR binding at low micromolar concentrations. Antiviral studies are also consistent with the in vitro and cell-based assays. MBOs provide a framework for the development of future RNA-targeting molecules.

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While chemists have excelled at the design and synthesis of organic molecules that inhibit protein functions by binding to active sites, there exists a lack of basic knowledge about how one should design a molecule to target a folded RNA.[1–4] The fact that proteins have folded, three-dimensional structures with unique binding pockets allows chemists to develop small organic molecules that bind with high affinity and specificity to a target protein. Because RNA can also have folded, three-dimensional structures, it should be possible for chemists to design new molecules that bind a target RNA with high affinity and specificity. A wealth of structural information on RNA has demonstrated that this biopolymer can adopt a multitude of folded structures.[5,6] In the cell, RNA often has folded structures to create protein or small molecule binding sites or to perform catalytic functions.[6] In many cases, the folded RNA structures approach the complexity of folded protein structures. Despite the amount of structural information, RNA continues to be underutilized as a target for drug development because there is a lack of synthetic RNA-binding molecules with well-defined molecular recognition properties associated with biological activity.[7]

The most common types of molecules that have been designed for RNA binding include aminoglycosides, polypeptides, and polycyclic aromatic molecules.[1] By incorporating a significant amount of cationic charge or aromatic density in each of these molecular types, excellent binding affinity to a target RNA can be achieved; however, affinity is usually attained at the expense of specificity for the target. Other approaches to identify RNA-binding molecules have explored high-throughput screening of chemical libraries (either in vitro or in silico).[8–12] While a few interesting leads from such studies have been identified, most results contribute more to the techniques of screening rather than identifying new molecular frameworks for development into RNA-binding drugs. The emerging picture from these pioneering studies indicates that new types of RNA-specific chemical scaffolds must be developed.[7]

We are studying the RNA-binding properties of a novel class of molecules termed Multivalent Binding Oligomers (MBOs). These molecules are derived from amino acids and their synthesis is similar to that of polypeptides. MBOs are oligomeric, like polypeptides, but an amine linkage holds the adjacent amino acids together instead of an amide bond. MBOs were designed to bind to RNA targets while avoiding the disadvantages of other classes of RNA-binding molecules. Specifically, MBOs contain sidechains with non-ionic functional groups to direct the specificity of binding through hydrogen bonding or aromatic–aromatic interactions, while the amines in the backbone contribute ionic interactions to facilitate binding to the anionic RNA backbone. At the same time, the presence of the sidechains is hypothesized to reduce the amount of non-specific binding to non-target RNAs when interactions between the sidechains and RNA are unfavorable.

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We report in this Letter the development and characterization of MBOs to target TAR, an HIV RNA that forms a stem-loop with a three nucleotide bulge (Fig. 1A).[13,14] The HIV protein Tat must bind to TAR for viral transcription to proceed efficiently, and molecules that inhibit the association of Tat with TAR block replication of the virus.[15] TAR is present in all HIV-1 transcripts, is highly conserved, and must fold into a stem-bulge-loop structure to be recognized by Tat.[16] Therefore, molecules that can bind with high affinity and selectivity to the bulge of TAR could evolve into new treatments for HIV infection.[17] Previously developed TAR-binding molecules have demonstrated that this is a good target to inhibit HIV,[18–21] but most of the currently available TAR binders are not suitable for further drug development due to non-specific binding,[22,23] toxicity,[24,25] poor biological activity (sometimes due to poor cellular uptake),[26] or a combination of these problems. The activity of a number of other TAR binders is still being investigated, and in some cases only limited characterization of biological activity has been performed.[27–30] The results presented below demonstrate that proper selection of side chains and oligomer length lead to MBOs that inhibit Tat–TAR association in vitro, are cell-permeable, maintain activity in a cell-based model system, and exert anti-HIV activity in infected white blood cells across a range of different clinically-derived strains of HIV-1. Structural data are also presented that confirm specific interactions between one of the most active MBOs and the bulge of TAR.

Trimer and cell-permeability. Our starting point to develop MBO inhibitors of Tat–TAR association was the molecule YYY (Fig. 1B), which is derived from tyrosines and binds to the bulge of TAR with moderate affinity (K_d ~5 μM). Previously, we had developed the chemistry to make such molecules and provided initial characterization of their binding to TAR.[31] Before developing more potent derivatives, YYY was investigated to ascertain if this type of molecule could be taken up by cells. Therefore, a fluorescein-labeled version of this molecule was synthesized (YYY-Fl), and cell-uptake studies were performed using HeLa cells. Fluorescence imaging showed reasonable cellular penetration, with concentration of the molecule in the nucleus and nucleolus (Fig. 1C). Based on these encouraging initial results, we felt that other MBO derivatives would similarly be able to permeate cells.

In vitro inhibition assays and optimization. Binding between TAR and Tat is an important part of HIV replication. Before studying MBOs in cell-based antiviral assays, the ability of MBOs to prevent Tat–TAR formation in vitro was optimized. Using an established competition assay,[32] a series of MBO derivatives were investigated for their ability to inhibit association between TAR and a fluorescently-labeled peptide derived from Tat. Each inhibition curve was fit to a single-site binding model to provide an EC_{50} value. This system was used as the principal method to evaluate the inhibitory potential of each MBO.

As shown in Table 1, the length of the MBO has a significant effect on inhibitory activity. For instance, the EC_{50} improves by two orders of magnitude as the length increases from a YYY trimer to a YYYYYYYY octamer. Next, the importance of side chains for inhibition of Tat binding was investigated using a series of MBO hexamers. Systematic replacement of a tyrosine side chain with alanine shows only modest decreases in activity, and there is no change in activity when this modification is introduced at positions 2 and 4 within the sequence. A derivative that consists of one tyrosine and five alanine side chains displays considerably weaker activity compared to hexamers composed mostly of tyrosines. To test whether MBOs can selectively inhibit Tat–TAR over another protein–RNA complex, an established competition assay that monitors Rev–RRE binding was used.[33–35] Using the fluorescence-based competition assay, the MBOs YYYYYY and YYYAYY displayed no inhibition of Rev binding to RRE up to an MBO concentration of 20 μM (see Supplementary data for details). Therefore, these two MBOs are at least 20 times more selective for inhibition of Tat–TAR over Rev–RRE.

Other amino-acid-derived side chains were incorporated into hexameric MBOs to determine their effects on inhibition of Tat–TAR binding. Lysine and tryptophan have commonly been used in peptides to improve RNA binding by increasing the amount of cationic charge or pi-stacking between the peptide and the RNA. Introducing one lysine sidechain at position 4 (YYYKYY) improved the activity three times compared to the analog made entirely from tyrosine, but incorporation of an additional lysine or a tryptophan sidechain did not further improve the activity. Since position 4 was amenable to sidechain variation in the MBO hexamer, the same

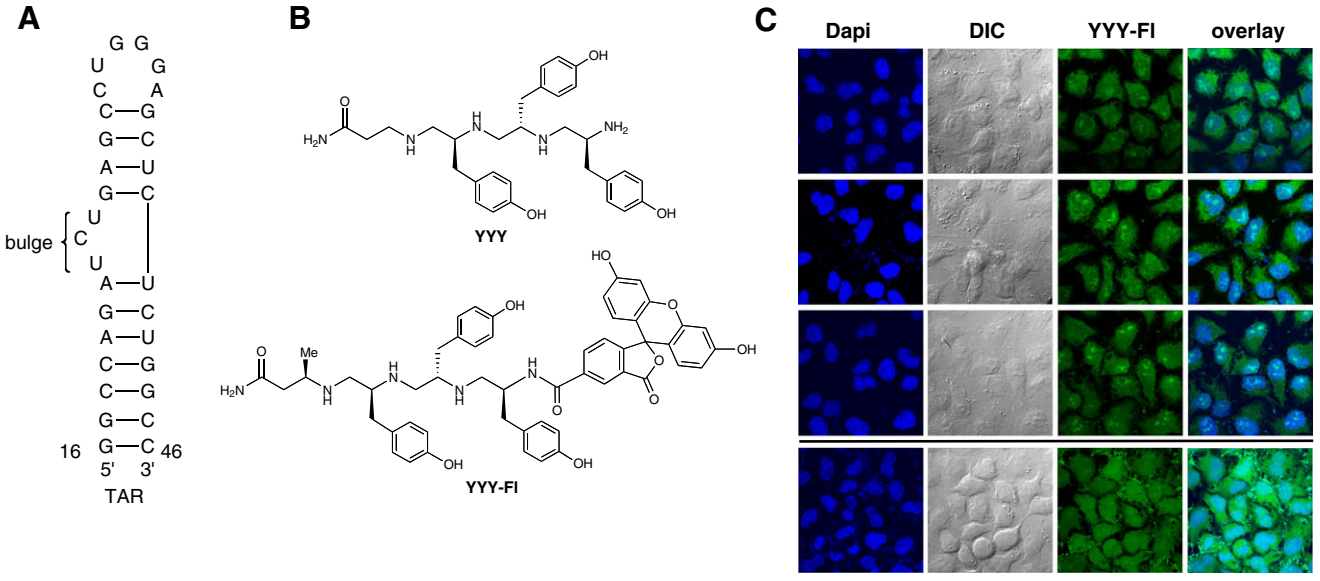

Figure 1. (A) Sequence and secondary structure of TAR. (B) Initial lead MBO (YYY) with K_d ~5 μM for binding to the bulge of TAR and the fluorescein-labeled derivative (YYY-Fl). (C) Three separate views of cellular uptake of YYY-Fl in HeLa cells (top three rows show 2 μM of YYY-Fl incubated for 30 min at 37 °C followed by washing, fixing, permeabilization and staining; bottom row shows results with fluorescein alone).

Table 1
EC_{50} values for MBO inhibition of Tat-peptide binding to TAR and for MBO-promoted aggregation of TAR in vitro (for fluorescence competition 100 nM TAR(49–57) and 100 nM Tat-peptide were used; aggregation observed by native gel electrophoresis using ^{32}P-labeled TAR (residues 17–45))

Length variation		Alanine scan		Sidechain variation		TAR aggregation	
MBO sequence	EC_{50} (μM) in vitro	MBO sequence	EC_{50} (μM) in vitro	MBO sequence	EC_{50} (μM) in vitro	MBO sequence	EC_{50} (μM) in vitro
YYY	83.8 ± 3.7	YAYYYY	1.9 ± 0.3	YYYKYY	0.5 ± 0.1		
YYYY	8.9 ± 1.0	YYAYYY	3.2 ± 0.4	YYYKWY	0.3 ± 0.1	YYYY	450 ± 20
YYYYY	3.0 ± 0.7	YYYAYY	1.6 ± 0.3	YKYKYY	0.4 ± 0.1	YYYYY	220 ± 10
YYYYYY	1.7 ± 0.4	YYYYAY	3.9 ± 0.5	YKYKWY	0.2 ± 0.1	YYYYYY	40 ± 10
YYYYYYY	0.5 ± 0.2	YYYYYA	3.3 ± 0.5	YYYAYYY	0.5 ± 0.1		
YYYYYYYY	0.2 ± 0.1	YAAAAA	14 ± 2	YYYAYYYY	0.20 ± 0.02		

Single letter amino acid codes are used to represent the sidechain, but the backbone for each entry is similar to the structures shown in Figure 1.

position was changed to an alanine in the corresponding heptamer and octamer (YYYAYYY and YYYAYYYY). Similarly, no detectable change in inhibition of Tat–TAR association was observed.

The MBOs of the current study all contain a polyamine backbone that may be protonated under the conditions of the binding assays and could cause non-specific RNA aggregation similar to other types of highly charged molecules. Using native gel electrophoresis with ^{32}P-labeled TAR (residues 17–45), the concentration at which representative MBOs induced TAR aggregation was determined (see Supplementary data). The studies showed that the aggregation of TAR is dependent on the length of the MBO; lower EC_{50} values were observed for longer MBOs (Table 1). Since the EC_{50} values for MBO-induced TAR aggregation are significantly higher than the IC_{50} values reported in Table 1, it is likely that the fluorescence-based competition assays correctly report specific inhibition of the Tat–TAR complex by MBOs.

Additional methods were employed to further characterize the association of the most active MBOs with TAR. Heteronuclear NMR spectroscopy has been used to examine the structure of HIV-1 TAR in both ligand-bound and free states.[14,36–38] Therefore, following the changes in chemical shifts of TAR as an MBO is titrated into solution provides an indication of how the MBO interacts with TAR. Upon titration of YYYAYY into TAR, the most significant chemical-shift changes were observed for residues in the bulge area of TAR (Fig. 2 and Supplementary Fig. S5), confirming that YYYAYY specifically interacts with this bulge. Increased line broadening of select NMR resonances is also in agreement with specific binding of YYYAYY to the bulge of TAR (Supplementary Fig. S5), but hampers high-resolution NMR structure determination of the complex. Titration of the YYY MBO leads to similar chemical-shift changes and line-broadening effects (not shown), further supporting that MBOs specifically target the Tat-binding area of TAR. Given the modest chemical-shift changes observed upon addition of YYY and YYYAYY, it is likely that binding of these MBOs does not significantly alter the structure of free TAR, in contrast with the binding of argininamide and small Tat-derived peptides that cause a major conformational change in the TAR bulge.[14,36,37]

Characterization of Tat–TAR inhibition in a cell-based model. An established method was used to investigate whether the in vitro inhibition of Tat–TAR formation by MBOs could be similarly observed in a cell-based assay, and the results guided which MBOs would be suitable for antiviral tests.[39] The assay directly probes for inhibition of Tat–TAR complex formation relative to non-specific binding. In this assay, HeLa cells have been modified such that the HIV-1 Tat gene and the firefly luciferase gene (expressed on a bicistronic mRNA), as well as an HIV-1 LTR-renilla luciferase reporter gene construct are stably integrated into the HeLa cell's DNA. The two different luciferase proteins represent reporter signals for HIV-1 Tat–TAR function (renilla) and non-specific toxicity (firefly), and comparison of these two different signals indicates a molecule's specificity for Tat–TAR inhibition. For instance, dose-dependent decreases in luminescence from renilla luciferase with no effect on firefly luciferase signal indicate that inhibition of Tat–TAR binding occurs, while decreases in luminescence from firefly luciferase indicate that either expression of the Tat protein is affected (presumably due to non-specific binding) or the compound is cytotoxic. The ideal inhibitor should decrease luminescence from renilla luciferase without affecting firefly luciferase. In these experiments, the IC_{50} is derived from the dose-dependent decrease in renilla luciferase while the TC_{50} comes from changes in

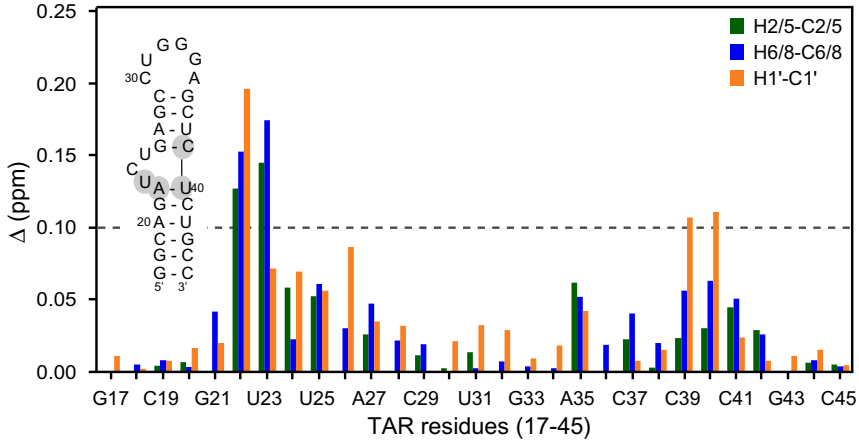

Figure 2. NMR chemical-shift mapping of YYYAYY binding on HIV-1 TAR. Several chemical-shift changes (Δ in ppm ±0.03 ppm; $\Delta = [(\Delta_H)^2 + (0.3 \times \Delta_C)^2]^{1/2}$) were observed after addition of 1 mM of YYYAYY to 1 mM ^{13}C/^{15}N-labeled TAR. Residues undergoing significant chemical-shift changes for the H1′–C1′, H6/8–C6/8, or H2/5–C2/5 atoms (>0.10 ppm) are circled in grey on the secondary structure of HIV-1 TAR.

Table 2
Activities of MBOs against Tat and Rev function in HeLa model cell systems (errors for IC_{50} values of tat function are in the Supplementary data)

MBO sequence	Tat function		Rev function	
	IC_{50} (μM)	TC_{50} (μM)	IC_{50} (μM)	TC_{50} (μM)
YYYY	19	>100	>100	>100
YYYYY	30	>100	>100	>100
YYYYYY	7	>100	34	>100
YYYAYY	6	>100	>100	>100
YYYKYY	12	>100	>100	>100
YKYKYY	35	38	—	—
YKYKWY	10	12	—	—
YYYAYYY	16	>100	60	>100
YYYAYYYY	0.9	2.4	—	—

firefly luciferase. An analogous system has also been established to monitor Rev–RRE interactions and was used as a specificity control.[40]

Results from the tests of several MBOs in the HeLa-derived model cell systems are shown in Table 2. The results demonstrate that the activity of the MBOs is ideal at the hexamer and heptamer lengths. In particular, YYYYYY and YYYAYY have IC_{50} values for the inhibition of Tat function in the low micromolar range with TC_{50} values above 100 μM in the same assay. In the tests for Rev function, YYYYYY showed activity at 34 μM while YYYAYY did not display any activity up to 100 μM. Therefore, YYYAYY appears to be highly selective. It is interesting to note that MBO hexamer variants with additional lysine sidechains or a tryptophan sidechain actually lost potency or selectivity despite observed improvements in the in vitro assay previously mentioned. For instance, YYYKYY is about three times more effective at inhibiting Tat–TAR binding than YYYYYY according to the in vitro fluorescence competition assay (Table 1), but its IC_{50} value is twice as large in the Tat function assay. Incorporation of an additional lysine (as in YKYKYY) eliminated specificity for inhibition of Tat–TAR binding compared to non-specific inhibition (IC_{50} and TC_{50} values were about the same). The heptamer YYYAYYY also has good activity, but it was less specific for Tat–TAR inhibition over Rev–RRE. An octamer displayed considerable non-specific activity in the Tat function assay.

Antiviral activity. Based on the results of the model cell system, three MBOs (two hexamers and a heptamer) were advanced into tests for antiviral activity against HIV-1 infection of peripheral blood mononuclear cells (PBMCs).[41] Antiviral tests with a laboratory-adapted strain of HIV-1 (Ba-L) showed all three MBOs had activity in the low micromolar range (Table 3). Based on these results, the study was expanded to include tests against clinical HIV-1 isolates (Clades A–O). All the MBOs displayed activity against each HIV-1 clade that was tested. While some variation is present between clades, the variability is not unexpected due to the heterogeneous nature of HIV (see discussion below). Toxicity levels of PBMCs to the MBOs were all significantly higher than the concentrations at which the molecules show activity against HIV-1.

We verified that the compounds were not preventing virus entry using a cell-based viral entry inhibition assay in TZM-bl cells.[42,43] None of the MBOs listed in Table 3 inhibited viral entry up to concentrations of 25 μM (Supplementary data).

Since the initial discovery of Tat–TAR association as an important part of HIV replication, there have been numerous attempts to block formation of this protein–RNA complex with synthetic molecules.[17] To date, only a few of the currently available inhibitors have the properties conducive to further development into a drug.[10,22,44–46] While research into new types of inhibitors for Tat–TAR association is ongoing, it seems that many initial strategies have been abandoned.[47] In our opinion, the lack of good drug candidates is part of a larger problem in which there exists a lack of molecular scaffolds designed for specific targeting of RNA.

The work presented in this report provides a good starting point to develop RNA-targeting molecules for TAR as well as other RNA structures. In this study, fluorescence studies indicate that MBOs bind the bulge of TAR over RRE and NMR studies corroborate the presence of binding interactions between MBOs and the TAR bulge. In contrast to other TAR ligands,[48,49] MBOs do not induce significant structural changes in the RNA. An interesting feature of the MBOs is that they behave as multivalent binders of TAR that rely on a combination of side chain and backbone interactions to achieve their binding and specificity. As shown from the alanine scan in Table 1, there is not a single side chain that provides the main driving force for Tat–TAR inhibition, but rather the combination of all side chains is essential. Our results indicate that a delicate balance of charge must be achieved to attain specific TAR binding in a cell-based system. While cationic side chains can be incorporated into an MBO to gain increased activity in vitro, the cell-based model system shows very rapid loss in activity or specificity with modest increases in MBO charge (possibly due to increased non-specific binding). The best inhibitors developed in this study (YYYYYY and YYYAYY) maintain consistent activity across in vitro tests, model cell-based systems, and also display similar anti-HIV activity.

While the specificity of the MBOs synthesized in these studies does not approach the levels seen for small molecule drugs that target proteins, there is currently no well-defined level of specificity for targeting HIV RNA. Since HIV is a highly variable and heterogeneous virus, molecules that are too specific may also be ineffective or not broadly effective. While the TAR sequence shown in Figure 1 appears in some HIV strains, there is variability in the sequence of the bulge from one virus clade to another.[50] The observation that the MBOs listed in Table 3 display antiviral activity against a number of different HIV clades indicates the MBOs retain their inhibitory activity despite the natural variance among different TAR sequences (Fig. 3). At the same time, toxicity of these MBOs to PBMCs did not appear problematic. While specificity is an important feature to be engineered into RNA-binding molecules, we also feel that complete specificity may not necessarily be required or desired when targeting an RNA sequence in HIV. In particular, the propensity of HIV to mutate indicates that a highly specific TAR-binding molecule may become rapidly ineffective due to the development of resistance. We also note that, according to the NMR studies, the MBO YYYAYY binds to the TAR bulge near residues that are conserved across clades. The MBOs developed in this study hopefully will provide a guide to future generations of RNA-binding molecules. While significant optimization is still

Table 3
Anti-HIV activity of MBOs against infection in PBMC (all values are IC_{50} values in μM)

MBO sequence	HIV-1 Ba-L	A UG/92/029	B HT/92/599	C 97ZA003	D UG/92/001	E CMU06	F BR/93/020	G JV1083	O BCF02
YYYYYY	2	6.7	8.8	27	6.0	19.0	4.7	13.1	18.9
YYYAYY	5	4.5	12.7	32	5.1	7.7	7.5	17.8	20.1
YYYAYYY	4	2.3	4.5	25	2.3	18.2	5.1	11.9	16.3

For the assays with HIV-1 Ba-L, the TC_{50} was 76, 56, 55 μM, respectively. For the assays with Clades A–O, the TC_{50} was >100 μM for the hexamer MBOs and 75 μM for the heptamer MBO (see the Supplementary data for the errors associated with the IC_{50} values).

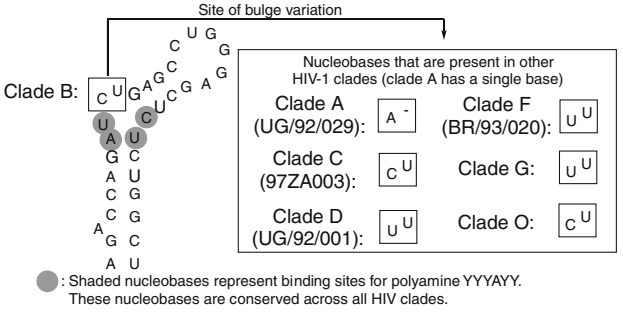

Figure 3. Points of variability within TAR compared with binding sites for YYYAYY (HIV sequence information obtained from the HIV Database maintained by Los Alamos National Laboratory (www.hiv.lanl.gov)).

needed for MBOs to become effective drugs, the nature of the MBO scaffold provides significant opportunities for modification and derivatization to target other RNA structures.

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Supplementary data

Supplementary data (synthetic procedures and data for all assays) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.10.078.

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