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Acylideneoxoindoles: A new class of reversible inhibitors of human transglutaminase 2

Cornelius Klöck [a], Xi Jin [a], Kihang Choi [a], Chaitan Khosla [a,b,*], Peter B. Madrid [c], Andrew Spencer [d], Brian C. Raimundo [e], Paul Boardman [e], Guido Lanza [e], John H. Griffin [e,*]

[a] Department of Chemistry, Stanford University, Stanford, CA 94305, USA
[b] Deparment of Chemical Engineering, Stanford University, Stanford, CA 94305, USA
[c] Department of Medicinal Chemistry, SRI International, Menlo Park, CA 94025, USA
[d] Celiac Sprue Research Foundation, PO Box 61193, Palo Alto, CA 94306-1193, USA
[e] Numerate, Inc., 1150 Bayhill Drive, Ste 203, San Bruno, CA 94066, USA

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ABSTRACT

Inhibitors of human transglutaminase 2 (TG2) are anticipated to be useful in the therapy of a variety of diseases including celiac sprue as well as certain CNS disorders and cancers. A class of 3-acylidene-2-oxoindoles was identified as potent reversible inhibitors of human TG2. Structure–activity relationship analysis of a lead compound led to the generation of several potent, competitive inhibitors. Analogs with significant non-competitive character were also identified, suggesting that the compounds bind at one or more allosteric regulatory sites on this multidomain enzyme. The most active compounds had K_i values below 1.0 μM in two different kinetic assays for human TG2, and may therefore be suitable for investigations into the role of TG2 in physiology and disease in animals.

Transglutaminase 2 (TG2), a ubiquitous member of the mammalian transglutaminase enzyme family, is found in intracellular as well as extracellular environments of many organs. In the presence of Ca^{2+} and the absence of guanine nucleotides, TG2 adopts an open, catalytically competent conformation, which activates γ-glutaminyl residues on proteins as acyl donors and cross-links these to ε-amino groups of lysyl residues. As a result, proteolytically resistant isopeptide bonds are formed between proteins. Hydrolysis of the γ-glutamylacyl-enzyme intermediate results in deamidation of the substrate.[1,2] TG2 is implicated in the pathogenesis of disorders including neurological diseases such as Huntington's, Alzheimer's and Parkinson's diseases, certain types of cancers and renal diseases, cystic fibrosis and celiac sprue,[3–8] and may therefore be a suitable therapeutic target for one or more of these conditions.[9] Consequently, small molecule modulators of in vivo TG2 activity are of pharmacological and medicinal interest.

Several classes of irreversible inhibitors of TG2 have been described thus far (Fig. 1).[2,10–15] More recently, three classes of reversible inhibitors have also been reported.[16–18] Here, we present a structure–activity relationship (SAR) analysis for a new class of reversible inhibitors of human TG2, the acylideneoxoindoles.

Isatin (indoline-2,3-dione) is an endogenous indole in mammals with a range of biological activities.[19,20] Our motivation to screen this natural product as a candidate TG2 inhibitor was guided by the hypothesis that the cyclic α-keto amide structure of isatin may mimic the γ-carboxamide group of TG2 substrates. α-Keto amides, including isatin analogs, are widely utilized as reversible inhibitors of cysteine-dependent proteases.[21] This led us to propose that isatin analogs may also be reversible inhibitors of the cysteine transglutaminase TG2. In preliminary screening efforts, isatin was found to be a weak, reversible inhibitor of human TG2 (IC_{50} >0.25 mM), and certain five-substituted analogs with electron-withdrawing functional groups were somewhat more active (IC_{50} = 65–450 μM for 5-chloro, 5-bromo, 5-iodo, and 5,7-difluoroisatin).

Using this information and data available for other classes of TG2 inhibitors, we built a ligand-based statistical model with which to identify new TG2 inhibitors. This model was used to screen ChemNavigator's iResearch library of commercially available compounds, and to prioritize compounds for acquisition and testing. Among these were a series of symmetrical isatin dimers (**1**–**6**), as well as three 3-acylidene-2-oxoindoles: indirubin (**7**), isoindigotin (**8**), and methyl ketone (**9**) (Table 1).

Using a standard glutamate dehydrogenase (GDH)-coupled deamidation assay with Cbz-Gln-Gly (ZQG) as the acyl donor substrate,[22] isatin dimers linked 6,6′ (**1**), 5,5′ (**2**, **3**), and 1,1′ (**4**, **5**) were

* Corresponding authors.
E-mail addresses: khosla@stanford.edu (C. Khosla), john@numerate.com (J.H. Griffin).

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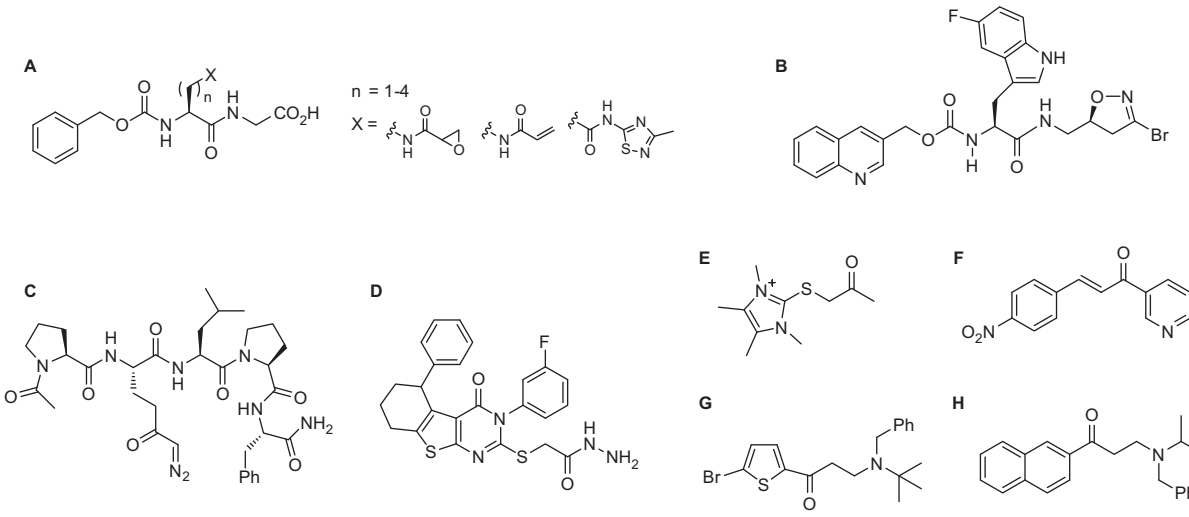


Figure 1. Selected TG2 inhibitors—irreversible dipeptide inhibitors (A),[11] irreversible DHI-based inhibitors (B),[10] irreversible DON-based substrate mimics (C),[2] reversible thienopyrimidinones (D),[16] irreversible imidazolium salts (E),[12,13] reversible azachalcones (F)[17] and aryl-β-aminoethylketones (G and H).[14,15]

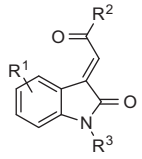


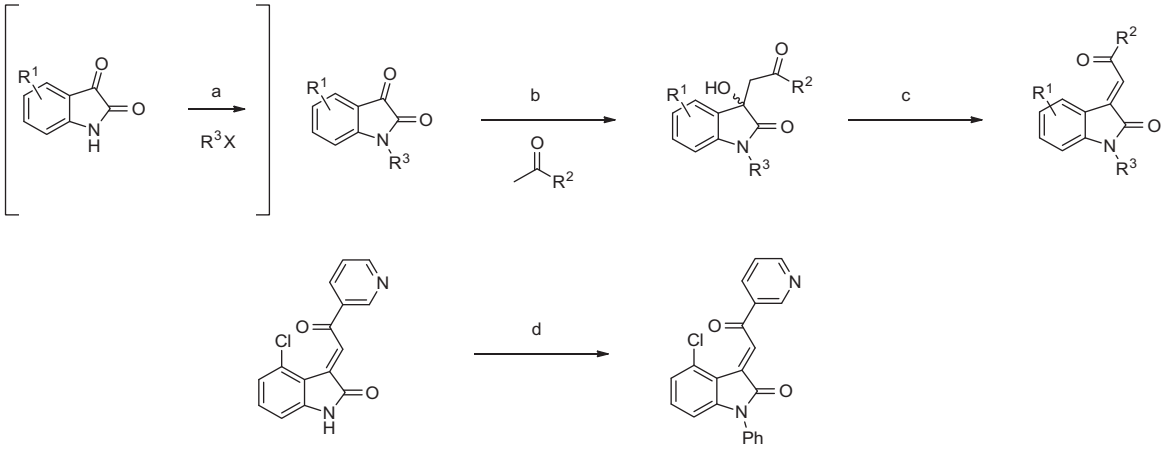
Figure 2. Acylidene oxoindoles.

found to display inhibition constants in the range of 18–40 μM, approximately 10-fold more potent than the simple 5-haloisatins. The linker can play a role in determining the activity of isatin dimers: the *m*-xylyl and methylene-linked analogs **4** and **6** were active whereas the *p*-xylyl linked analog **5**, a constitutional isomer of **4**, was not. Among the 3-acylidene oxoindoles, indirubin (**7**) was inactive, but isoindigotin (**8**) and the *E*-methyl ketone **9** proved to be promising inhibitors.

To explore the potential of acylidene oxoindoles as TG2 inhibitors, we undertook the synthesis of analogs of compound **9** bearing substitution in three regions—on the aromatic oxoindole ring (R^1), at the methyl position of the ketone (R^2), and on the amide nitrogen (R^3) (Fig. 2).

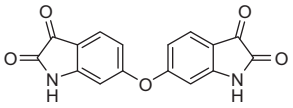
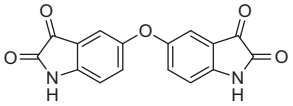
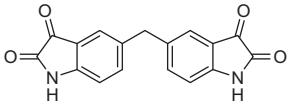
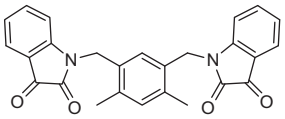
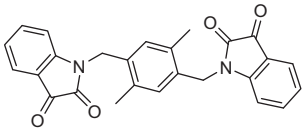
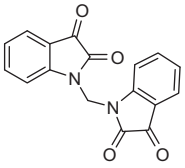
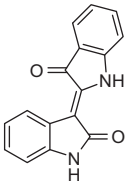
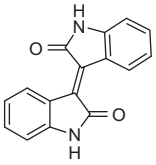
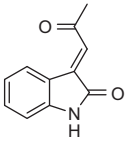
The acylidene oxoindoles were prepared by a two-step condensation–dehydration sequence from isatin or a substituted isatin along with acetone or an aryl methyl ketone (Scheme 1). The first step, performed under basic conditions, afforded β-hydroxy ketones which were isolated and then dehydrated under acidic conditions, or via the agency of methane sulfonyl chloride in pyridine, to produce the acylidene oxoindole.[23] All compounds were obtained as a single stereoisomer, which was assigned as the (*E*)-diastereomer based on the ^{1}H NMR spectra, which displayed downfield chemical shifts for the aromatic C-4 proton resonances.[24,25] N-Substituted compounds were prepared either via condensation–dehydration starting from the corresponding N-substituted isatin or via copper-mediated N-arylation of an acylidene oxoindole.[26]

The inhibitory properties of the acylidene oxoindoles toward TG2-catalyzed deamidation of ZQG were initially characterized using the GDH-coupled assay (Table 2). We first examined a small series of analogs of parent compound **9** bearing fluoro, chloro or ether substituents at the 4-, 5-, 6- or 7-position of the oxoindole system (compounds **10**–**14**). Here, the 4-chloro analog **10** exhibited the highest potency, with an IC_{50} value of 1.5 μM and a K_i value of 0.7 μM.



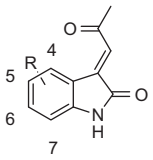
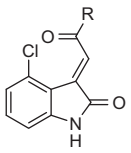
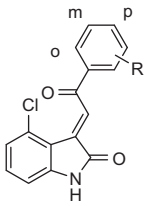
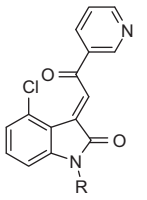
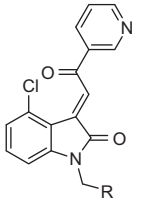
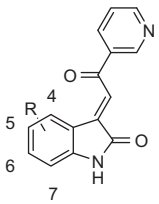
Scheme 1. Synthesis of 3-acylidene-2-oxoindoles. Top: synthesis of N1-H or N1-substituted analogs via condensation–dehydration of N1-H or N1-substituted isatins. Bottom: synthesis of N1-substituted analogs via N-arylation of N1-H compounds: Reagents and conditions: (a) R^3X (alkyl bromide or iodide), K_2CO_3, DMF, 16–48 h; (b) acetone, $NHEt_2$, 60 °C, 16 h or aryl methyl ketone, $NHEt_2$, EtOH, rt, 2–48 h; (c) HCl, AcOH, reflux, 0.5 h or HCl, AcOH, rt, 16 h; (d) $PhB(OH)_2$, $CuSO_4{\cdot}5H_2O$, pyridine, DCM, 16 h.

Table 1
Structures and TG2 inhibitory characteristics of isatin dimers and analogs

	Compd	IC_{50} (μM)	K_i (μM)
	**1**	30–40	–
	**2**	25	3
	**3**	30	15
	**4**	40	11
	**5**	>250	–
	**6**	18	10
	**7**	>100	–
	**8**	8	41
	**9**	11	10

Enzyme inhibition was measured using the coupled GDH assay ([TG2] = 0.5 μM). For IC_{50} values, the substrate was used at its K_m = 10 mM. The errors were typically less than 10%.

Table 2
Structures and activities of 3-acylidene-2-oxoindole inhibitors modified in all regions

	Compd	R =	IC_{50} (μM)	K_i (μM)
	**10**	4-Cl	1.5	0.7
	11	5-Cl	4.3	0.9
	12	6-F	11	5.4
	13	6-OCF_3	3.6	
	14	7-Cl	4.7	
	**15**	2′-Pyridyl	1.1	
	16	3′-Pyridyl	0.9	1.3
	17	4′-Pyridyl	1.2	
	18	3′-Pyr-5′-Br	1.4	
	19	3′-Pyr-4′-OMe	1.4	
	20	Ph	2.8	3.3
	**21**	*m*-Cl	0.7	
	22	*p*-Cl	0.8	
	23	*o*-OMe	0.9	
	24	*m*-OMe	1.0	
	25	*p*-OMe	1.1	
	26	*m*-NH_2	1.4	
	27	*p*-NH_2	1.1	
	**28**	Me	4.0	4.0
	29	*i*Pr	1.4	
	30	*i*Bu	1.0	
	31	Ph	0.8	
	**32**	Cyclohexyl	2.1	
	33	Ph	1.5	0.41
	34	CH_2Ph	1.5	
	35	$(CH_2)_2Ph$	1.3	
	36	$CONMe_2$	5.5	
	37	CO_2Et	1.8	
	38	(*m*-CO_2Me)Ph	1.1	
	39	(*p*-CO_2Me)Ph	1.2	
	**40**	H	22	12
	41	4-Br	0.9	0.4
	42	5-Cl	6.0	
	43	5-Br	4.8	3.0
	44	5-Me	6.3	
	45	5-NO_2	7.9	
	46	6-Cl	2.1 (70%)[a]	5.3
	47	6-Br	1.5 (35%)[a]	
	48	7-Cl	7.3	
	49	7-Br	2.9	

Their inhibitory characteristics against TG2 were measured in the GDH assay ([TG2] = 0.5 μM).
For IC_{50} values, the substrate was used at its K_m = 10 mM. The errors were typically less than 10%.
[a] If partial inhibition was observed, the maximum inhibition effect is shown in parentheses.

Using compound **10** as a new lead, we next examined the effects of replacing the methyl group on the ketone moiety with aromatic groups (compounds **15**–**27**). We observed that phenyl, substituted phenyl, pyridinyl, and substituted pyridinyl groups were well-tolerated at this position, affording compounds with IC_{50} values very similar to that of compound **10**. Negligible differences in TG2 inhibitory potency were observed among regioisomeric pyridin-2-yl, -3-yl or -4-yl analogs (**15**–**17**), 4′- or 5′-substituted pyridin-3-yl analogs (**18**, **19**), phenyl analog **20**, or phenyl analogs substituted at the 2′-, 3′- or 4′-positions with chloro, methoxy or amino groups (**21**–**27**).

The 4-chloro-pyridin-3′-yl analog **16** was used as a scaffold to examine the effects of substitution at the oxoindole nitrogen (compounds **28**–**39**). While methyl and dimethyl acetamido substituents led to 4- and 6-fold losses in potency for compounds **28** and **36**, respectively, a range of other substituents afforded analogs that were essentially equipotent to **16**. These substituents included primary and secondary alkyl (**29**, **30**, **32**), phenyl (**31**), 2-phenylethyl (**34**), 3-phenylpropyl (**35**) benzyl (**33**, **38**, **39**), and ethyl acetyl (**37**) groups.

We also used compound **16** as a starting point from which to examine more systematically the effects of substitution on the

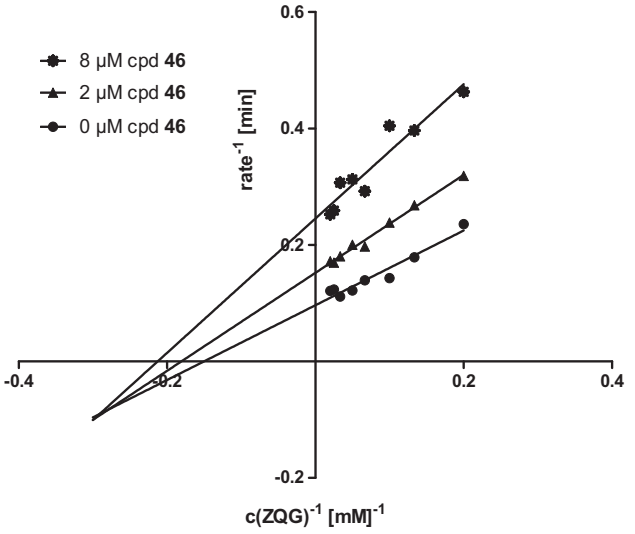


Figure 3. Lineweaver–Burk analysis of the 6-chloro substituted compound **46**, confirming its non-competitive inhibitory character. Reversibility was assigned based on the observation that preincubation of TG2 with inhibitors in the presence of 4 mM Ca^{+2} for 40 min did not appreciably alter the outcome of inhibition experiments (data not shown).

oxoindole benzene ring (compounds **40**–**49**). Removal of the 4-chloro substituent led to a >20-fold loss in potency for compound **40**, but replacing chlorine with bromine at the 4-position afforded the sub-micromolar inhibitor **41**. Analogs bearing chloro, bromo, methyl or nitro groups at the 5-position (**42**–**45**), or with chloro or bromo groups in the 7-position (**48**, **49**), displayed IC_{50} values in the range of 2.9–7.9 µM, some 3- to 9-fold less potent than compound **16**. The 6-chloro and 6-bromo analogs **46** and **47** were nearly as potent as compound **16**. Interestingly, these compounds were only partial inhibitors of TG2, achieving maximal inhibition of only 70% and 35%, respectively. This behavior suggests a non-competitive, allosteric mode of action, a hypothesis that was confirmed for compound **46** via Lineweaver–Burk analysis (Fig. 3).

The GDH-coupled assay for TG2 activity became problematic as the potencies of acylidene oxoindole inhibitors increased. The typical enzyme concentration used in this assay is 0.5 µM, which precludes measurement of K_i values below this concentration. Therefore, a more sensitive direct fluorometric assay was implemented, which follows the TG2-catalyzed release of 7-hydroxycoumarin from an ester substrate and allows the use of enzyme concentrations below 100 nM.[27] Table 3 presents the IC_{50} values obtained for 43 acylideneoxoindole inhibitors with this method and with the GDH-coupled assay, where it may be seen that the fluorometric assay indeed allowed us to determine lower IC_{50} values (range 0.090–20 µM) than did the GDH-coupled assay (range 0.8–22 µM). The fluorometric assay also uncovered partial inhibition activity with a larger subset of compounds, including compounds **19**, **22**, **27**, **47**, and **48**. Furthermore, using a mixed inhibition model for analyzing the observed inhibition kinetic behavior, non-competitive binding character could be detected for a number of compounds that did not exhibit partial inhibition towards TG2, including compounds **33**, **41**, and **43** (Table 4).

Acylidene oxoindoles have been widely utilized as synthetic intermediates, for example, in the synthesis of stereochemically rich and diverse spirocycles,[28–32] natural products[33] and chemical libraries.[34] Acylidene oxoindoles have also shown biological activity, for example, as kinase inhibitors,[35,36] phosphatase inhibitors,[37] promoters of vasodilatation,[38] antifungal agents,[39] and activators of TRP1 ion channels.[40] As described in this communication, we have identified in 3-acylidene-2-oxoindoles a new class of reversible inhibitors of human TG2. Through synthesis and biochemical

Table 4
Analysis of inhibition constants of selected 3-acylidene-2-oxoindolesin the GDH assay ([TG2] = 0.5 µM) and the fluorescent assay ([TG2] = 15 nM), α is the ratio K_i'/K_i in a mixed inhibition model

Compd	GDH-coupled assay		Fluorescent assay	
	K_i (µM)	α	K_i (µM)	α
10	0.7	>100		
11	0.9	>100		
12	5.4	>100		
16	1.3	>100		
20	3.3	>100		
28	4.0	>100		
33	0.41	1.8	1.0	3.6
40	12	>100		
41	0.4	>100	0.25	2.6
43	3.0	>100	0.68	4.4
46	5.3	0.86		

The larger α, the more competitive is the inhibition; when α = 1, the inhibition is simple non-competitive and when α < 1, the inhibition is increasingly uncompetitive.

Table 3
Comparison of IC_{50} concentrations as determined by the widely used coupled GDH assay and the coumarin-based fluorescence assay with a better dynamic range for submicromolar values

Compd	GDH IC_{50} (µM)	Fluoresc. IC_{50} (µM)	Compd	GDH IC_{50} (µM)	Fluoresc. IC_{50} (µM)	Compd	GDH IC_{50} (µM)	Fluoresc. IC_{50} (µM)
9	11	19	**23**	0.9	0.74	**37**	1.8	2.5
10	1.5	–	**24**	1.0	1.1	**38**	1.1	5.6
11	4.3	3.7	**25**	1.1	1.3	**39**	1.2	0.66
12	11	20	**26**	1.4	1.8	**40**	22	8.5
13	3.6	2.6	**27**	1.1	3.0 (65%)[a]	**41**	0.9	1.0
14	4.7	3.9	**28**	4.0	11	**42**	6.0	1.2
15	1.1	0.89	**29**	1.4	1.5	**43**	4.8	0.56
16	0.9	1.7	**30**	1.0	4.1	**44**	6.3	4.5
17	1.2	1.0	**31**	0.8	0.57	**45**	7.9	1.7
18	1.4	0.97	**32**	2.1	6.7	**46**	2.1 (70%)[a]	20
19	1.4	0.95 (50%)[a]	**33**	1.5	0.36	**47**	1.5 (35%)[a]	0.36 (65%)[a]
20	2.8	20	**34**	1.5	2.9	**48**	7.3	1.6 (70%)[a]
21	0.7	2.8	**35**	1.3	1.0	**49**	2.9	3.2
22	0.8	0.09 (60%)[a]	**36**	5.5	9.2			

For IC_{50} values in the GDH-coupled assay, the substrate was used at its K_m = 10 mM with 500 nM TG2. In the fluorescent assay, IC_{50} values were measured at 10 µM substrate concentration (=2 * K_m) with 15 nM TG2. The errors were typically less than 10%.

[a] If partial inhibition was observed, the maximum inhibition effect is shown in parentheses.

characterization of analogs substituted on the oxoindole benzene ring, at the exocyclic ketone group and on the oxoindole nitrogen, we have demonstrated that (1) TG2 inhibitory potency is increased by substitution of chlorine or bromine at position C-4, and (2) a range of aryl substituents may be attached to the ketone group, and a range of substituents may be added to oxoindole nitrogen with minimal impact on TG2 inhibitory potency. We have furthermore provided kinetic evidence that some acylidene oxoindoles are allosteric inhibitors of TG2 with a non-competitive inhibition mechanism. The most active compounds in this series inhibit TG2 at submicromolar concentrations, and may therefore be suitable for investigations into the role of TG2 in physiology and disease.

Acknowledgment

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.12.037.

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