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Synthesis and in vitro evaluation of anti-inflammatory activity of ester and amine derivatives of indoline in RAW 264.7 and peritoneal macrophages

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ABSTRACT

A prolonged increase in pro-inflammatory cytokines, TNF-α and IL-6 occurs in inflammatory diseases. Although existing therapies like steroids and TNF-α antagonists are effective they may cause serious adverse effects. We describe the preparation and evaluation for anti-inflammatory activity of 11 novel derivatives of indoline carbamates with a propionic ester, 2-aminoethyl, 3-aminopropyl 2-(dimethylamino)ethyl or 3-(dimethylamino)propyl group in positions 3 or 1. Compounds **25**, **26** and **29** were previously shown to inhibit acetylcholinesterase with IC_{50s} ranging from 0.4 to 55 μM and to prevent cytotoxicity induced by reactive oxygen species in a concentration range of 100 pM–1 μM. Compounds **25**, **26**, **29**, **9**, **10**, **17** and **18**, reduced NO, TNF-α and IL-6 at concentrations of 1–10 pM in LPS-activated RAW-264.7 and mouse peritoneal macrophages. The reduction in cytokines by compound **25** was associated with an increase in IκBα degradation and a decrease in the phosphorylation of p38 but not that of ERK. *Conclusion*: Indoline derivatives substituted at position 3 with chains carrying ester or amino groups may have potential for the treatment of chronic inflammatory and neurodegenerative diseases.

Inflammation is a normal response to infection and injury and involves the recruitment of immune systems to neutralize invading pathogens, repair injured tissues and promote wound healing.[1] Chronic or excessive activation of the immune system is associated with an increase reactive oxygen species (ROS), prolonged activation of inducible NO synthase (iNOS) and of the release of pro-inflammatory cytokines.[2,3] This may increase susceptibility to infections and cause ulcerative colitis (UC), rheumatoid arthritis (RA),[4] diabetes,[5] and cancer.[6] Drugs that block the action of the cytokine, tumor necrosis factor-α (TNF-α) have proved to be very effective in the treatment of UC and RA.[7–9] However, their chronic use may increase the risk of serious adverse effects,[10–14] and has resulted in the search for other therapies that prevent the excessive activity of cytokines and ROS without interfering with their normal function.

Indole-3-propionic acid (IPA) is a metabolite of 5-hydroxytryptamine that acts as an oxygen radical scavenger[15] and can protect cells against oxidative stress.[16] We found that the introduction of an OH into the 4 or 6 position of IPA increases the antioxidant ability which is further enhanced by its conversion to a carbamate.[17] However, the carbamate derivatives of IPA are much less water soluble than their corresponding indolines which were also more effective as oxygen radical scavengers and in conferring cytoprotection against oxidative stress.[17] The naturally occurring indole, melatonin has an amido side chain and has both antioxidant[18,19] and anti-inflammatory activity in vitro[20] and in vivo.[21]

Recognition of conserved products unique to micro-organisms such as lipopolysaccharide (LPS) occurs through activation of cell surface receptors–toll-like receptors (TLRs). This initiates a cascade of intracellular responses culminating in the release of NO and cytokines.[22] In the current study, we describe the evaluation of the potential anti-inflammatory activity of several carbamate derivatives of indoline propionic esters previously described.[17] We also synthesized novel indoline carbamates with a propionic ester, 2-aminoethyl, 3-aminopropyl, 2-(dimethylamino)ethyl or

Abbreviations: AD, Alzheimer's disease; TLRs, toll-like receptors; IPA, indole-3-propionic acid; iNOS, inducible NO synthase; DMEM, Dulbecco's modification of Eagle's medium; FCS, fetal calf serum; MTT, 3-(4,5-dimethyl-1,3-thiazol-2-yl)-2,5-diphenyl-2*H*-tetrazole-3-ium bromide; PMSF, phenyl methyl sulfonyl fluoride; RA, rheumatoid arthritis; ROS, reactive oxygen species; UC, ulcerative colitis; AP-1, activator protein 1; MAPKs, mitogen activated protein kinases; ERK, extracellular signal-regulated kinase; JNK, c-jun NH_2-terminal kinase.

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3-(dimethylamino)propyl group in the 3 or 1 positions and evaluated their anti-inflammatory activity in macrophages.

The syntheses of the various novel indole and indoline derivatives and their intermediates are described in Scheme 1. The final derivatives (some of which were described earlier)[17] were evaluated for anti-inflammatory activity as described below. Table 1 lists the novel compounds and those that were synthesized previously.[17] Starting materials **1** and **6** are commercially available and some of the early intermediates have been reported in the literature. In our previous paper[17] most of the studied derivatives possessed side chains that carried carboxylate functional groups, whereas latter compounds have side chains substituted with amino groups. All the indolinic compounds, which were evaluated for their biological activity, were isolated as the corresponding acid addition salts.

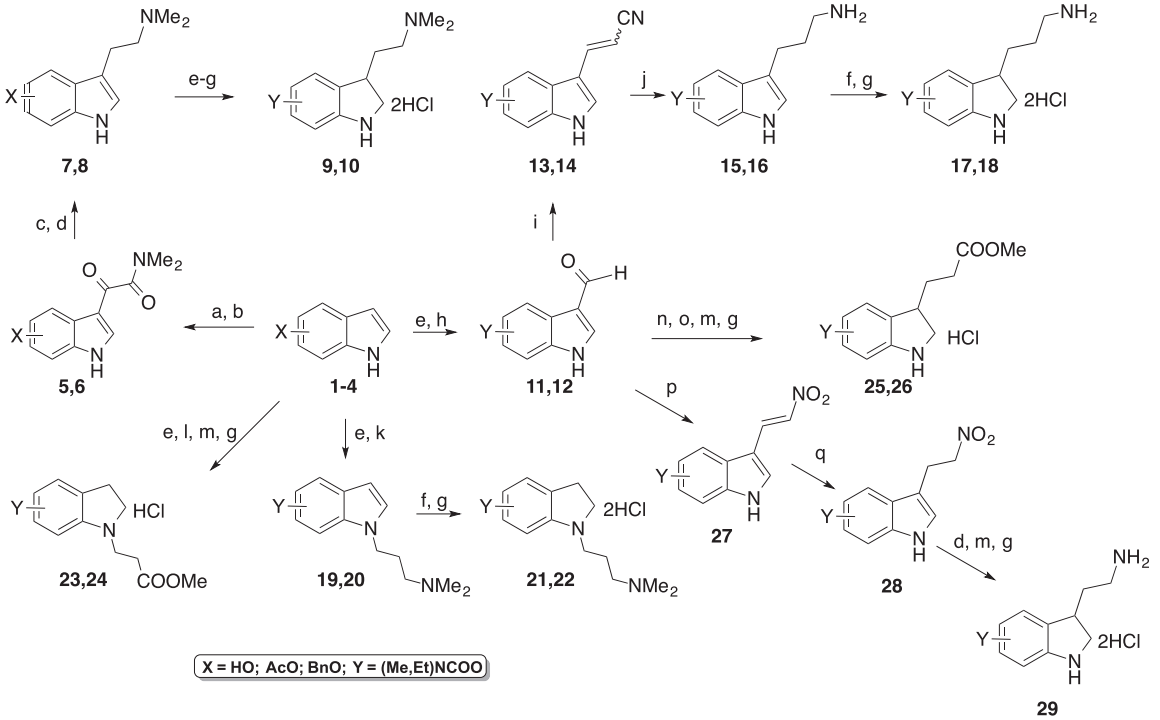
The 2-dimethylaminoethyl-substituted indolines **9** and **10** were prepared by reduction of the corresponding oxamides **5** and **6**,[23] which were debenzylated to give indoles **7** and **8**, that were then carbamoylated[24] and reduced to indolines with Et_3SiH/TFA.[25,26] It should be noted that in the course of the indole-to-indoline reductions carried out in TFA, both here as well as in the preparation of compounds **17** and **18**, small amounts of side-products having a CF_3CH_2-group attached to the ring-nitrogen were also obtained.[27,28] These minor side-products, which are not shown in Scheme 1 or in Table 1, were removed in the course of the purification of the indolines. The intermediates **13** and **14** were prepared by a Knoevenagel condensation between the corresponding aldehydes **11** and **12** and cyanoacetic acid.[29] The acrylonitrile side-chain was reduced by catalytic hydrogenation over PtO_2[30] to give indoles **15** and **16**, which were further reduced with Et_3SiH/TFA,[25,26] to give the indolines **17** and **18** initially isolated as TFA salts that were subsequently converted into the corresponding hydrochlorides. The N-alkylated indoles **19** and **20** obtained from **1** and **4** upon reaction with $Me_2NCH_2CH_2CH_2Cl$/NaH,[31] were reduced with Et_3SiH/TFA,[26] to give the TFA salts of the indolines, that were subsequently converted in the hydrochlorides **21** and **22**. Compounds **23**–**29** were described earlier[17] and are incorporated into Scheme 1 since some of their biological activities are described herein.

Table 1
Indolic and indolinic derivatives

Compound	X	Position of X
1	HO	4
2	HO	6
3	AcO	4
4	BnO	6
5	AcO	4
6	BnO	6
7	HO	4
8	HO	6
9–**29**	(Et,Me)NCOO	4 or 6

In each pair of compounds **9**–**29** the one with the odd number has the carbamate at position 4 and the one with the even number has the carbamate at position 6.

NO release was measured (as $NaNO_2$) in RAW 264.7 macrophages activated by LPS (5 μg/mL) from *Escherichia coli* (Sigma Ltd),[32] and cytokines were measured in peritoneal macrophages activated by LPS 1 μg/mL.[33] Figure 1 shows the concentration of each of the indoline derivatives that maximally reduced NO in RAW 264.7 macrophages and TNF-α and IL-6 in peritoneal macrophages, in comparison with the steroid budesonide and the naturally-occurring indole melatonin. Higher concentrations than those shown were either not more, or even less effective. In RAW 264.7 macrophages all the compounds were 10–1000 times more potent than budesonide and some were at least 100 times more potent than melatonin in reducing NO. Irrespective of the position of the side chain, compounds with a carbamate in position 4 (**9**, **17**, **25**) tended to be more effective than those with the carbamate in position 6, (**10**, **18**, **26**) except compound **21** which was less effective than compound **22** (Fig. 1A). Substitution of position 1 with



Scheme 1. Synthesis of indolines and their synthetic intermediates. Reagents and conditions: (a) $(COCl)_2$; (b) Me_2NH; (c) $LiAlH_4$; (d) H_2/Pd/C 10%; (e) (Me,Et)NCOCl; (f) Et_3SiH, TFA; (g) HCl; (h) $POCl_3$/DMF; (i) $HOOCCH_2CN$; (j) H_2/PtO_2/EtOH; (k) $ClCH_2CH_2NMe_2$·HCl/NaH/DMF; (l) CH_2=CH-COOMe/DBU/CH_3CN; (m) $NaBH_3CN$/AcOH or $NaBH_4$/TFA; (n) $HOOCCH_2COOMe$/Py/piperidine; (o) 10% Pd/C/HCO_2NH_4; (p) $MeNO_2/NH_4OAc$; (q) $NaBH_4$/silica gel/*n*-PrOH.

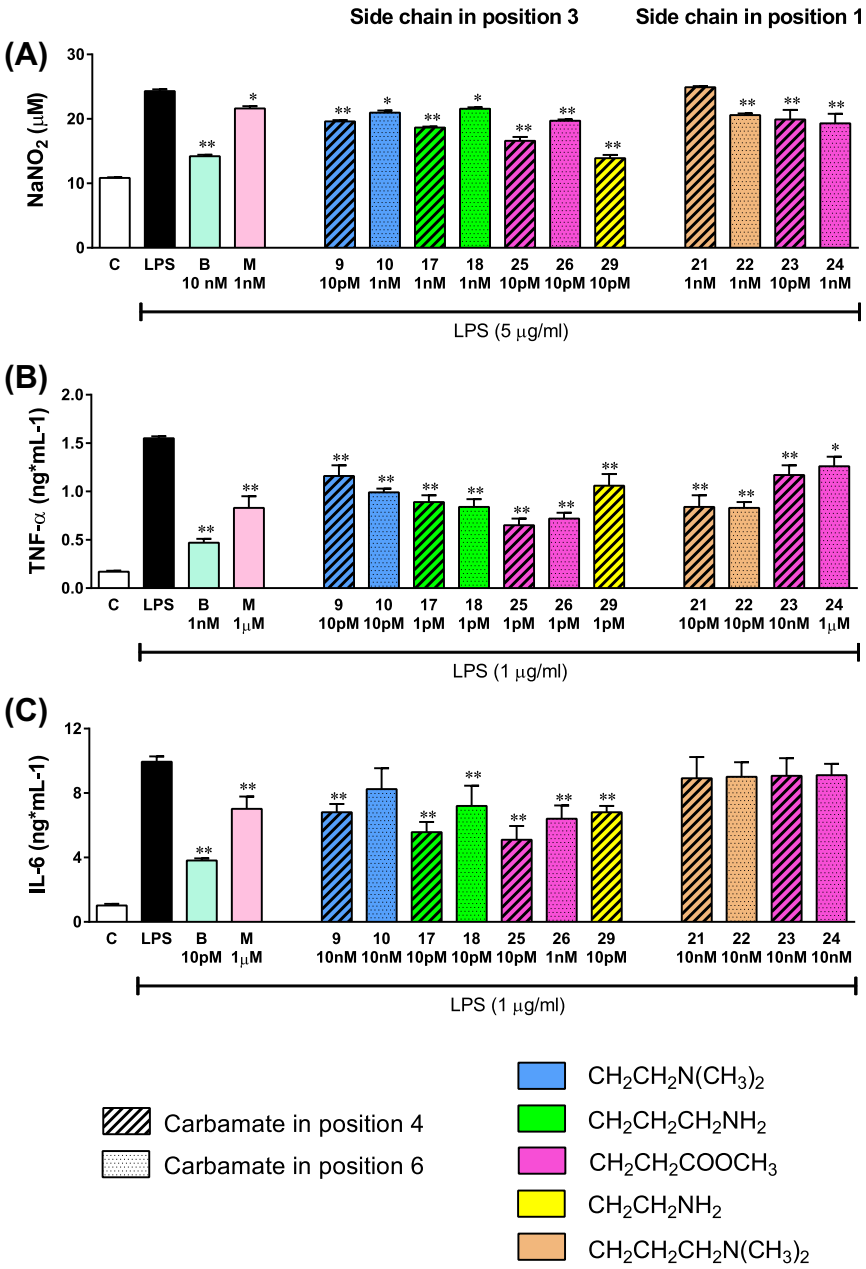


Figure 1. Reduction by novel indoline derivatives of NO and cytokines induced by LPS in macrophages. Data show the concentrations of compounds expressed in terms of the salt that caused the greatest reduction in NO or cytokines. The results are presented as the mean ± SEM from 6 measurements per compound in three independent experiments. Statistical differences between data from cells treated with LPS alone and those with compounds were analyzed by Analysis of Variance using SPSS statistical package version 19. A p value of <0.05 was considered to be significant. Each bar represents the mean ± SEM. (A) Compounds were administered 0.5 h prior to exposure to LPS (5 μg/mL) and measurements of NO (as $NaNO_2$) were made in RAW 264.7 cells after 24 h. Budesonide (B) was used as a positive control for these and subsequent experiments. M = melatonin. (B) TNF-α-Measurements were made in peritoneal macrophages 6 h after addition of LPS. (C) IL-6-Measurements were made in peritoneal macrophages 24 h after addition of LPS. Significantly different from LPS alone, $^{*}p$ <0.05; $^{**}p$ <0.01.

the propionic ester resulted in a reduction in activity, particularly if the carbamate was in position 6 (compound **24**).

In peritoneal macrophages, the most potent inhibitors of TNF-α were those with a 3-aminopropyl or propionic ester substituent, (**17**, **18**, **25**, **26**) with the carbamate either in position 4 or 6. Attaching the propionic ester substituent to position 1 resulted in a significant reduction in activity (**23**, **24**). Compounds **21** and **22** with a 3-(dimethyl amino) propyl side chain attached to position 1 were less potent than compounds **17** and **18** with a 3-aminopropyl or **25** and **26** with a propionic ester group at position 3 (Fig. 1B).

Budesonide reduced IL-6 at much lower concentrations than those affecting NO or TNF-α, but the indoline derivatives inhibited IL-6 at higher concentrations than those that inhibited TNF-α. The most potent inhibitors among the indoline carbamates had a 3-aminopropyl or propionic ester in position 3 and a carbamate in position 4 (**17**, **25**). Substitution of the propionic ester in position 1, (compound **23**) resulted in a complete loss of inhibitory activity against IL-6. Compounds **9** and **10** with a (2-dimethylamino)ethyl-group at position 3, or compounds **21** and **22** having a (3-dimethylamino)propyl-group at position 1 were much less active or inactive as inhibitors of IL-6 (Fig. 1C). Our data differ from those described by Liu et al.,[34] who found that many of the prenylated mono-carbonyl derivatives of curcumin were effective in reducing IL-6 but not TNF-α in RAW 264.7 macrophages. Since blockade of

TNF-α is considered to be the most effective treatment of RA and UC,[7–9] the indoline derivatives may be more efficacious than those of curcumin in treating chronic inflammatory conditions. Treatment of RAW 264.7 and peritoneal macrophages with all the compounds in concentrations ranging from 10 pM to 10 μM had no effect on cell viability as measured by the 3-(4,5-dimethyl-1,3-thiazol-2-yl)-2,5-diphenyl-2*H*-tetrazole-3-ium bromide (MTT) assay[35] (data not shown).

Binding of LPS to TLR4 in macrophages involves the activation of multiple signal transduction pathways, including mitogen activated protein kinases (MAPKs) such as p38, extracellular signal-regulated kinase (ERK) or c-jun NH_2-terminal kinase (JNK) which activate the transcription factor activator protein 1 (AP-1) leading to the production of pro-inflammatory entities.[36] Another pathway activated by LPS via TLR4 involves the phosphorylation of nuclear factor κB-light polypeptide gene enhancer inhibitor (IκB) and the degradation of IκBα that exposes nuclear localization signals on the p50/p65 complex, resulting in nuclear translocation of NF-κB.[37] The latter binds to specific regulated sequences in the DNA, thus controlling gene transcription, stimulation of iNOS to form NO and release of TNF-α and IL-6.[38] Pretreatment of LPS-stimulated peritoneal macrophages with compound **25** (100 pM), one of the most potent inhibitors of NO, TNF-α and IL-6, inhibited the phosphorylation of p38 but not that of ERK (Fig. 2) or JNK (not shown) assessed as described in.[39] Compound **25** also reduced the degradation of IκBα, which would be expected to decrease the nuclear translocation of NF-κB and explains how the compound reduces NO and cytokines in cells activated by LPS.

It has been suggested that neuroinflammation occurs in Alzheimer's disease (AD).[40] Although the acetylcholinesterase (AChE) inhibitor, donepezil currently used for treatment of this condition was shown to exhibit anti-inflammatory activity in cells, this was seen at much higher concentrations than those that inhibit AChE,[41] making it unlikely that it contributes to the therapeutic effect of the drug. The indoline-3-propionic esters containing a carbamate moiety were previously shown to inhibit both AChE and butyrylcholinesterase (BuChE).[17] It was therefore of interest to see whether the novel compounds with an amino group in the side chain also inhibited AChE and BuChE at similar or lower concentrations than those which showed anti-inflammatory activity. AChE and BuChE inhibitory activity was assessed as previously described[17] and is depicted in Table 2 as IC_{50}s (concentrations required to reduce enzyme activity by 50%). Compounds **9**, **17**, **21** and **29** with amino groups and carbamates at position 4 were found to be more potent inhibitors of AChE than compounds **25** and **26** with a propionic ester. The increased activity may result from the interaction of the basic group with the peripheral anionic site situated at the entrance to the narrow gorge of the enzyme which could improve the alignment of the molecule to the catalytic site situated near its base, as previously suggested.[17] For all pairs of compounds, those with a carbamate in position 4 were 13–67 times more potent as ChE inhibitors than their analogs with the carbamate in position 6, irrespective of the nature of the side chain. The reduced activity of all the compounds having the carbamate at position 6 compared to those with a carbamate at position 4, may stem from a hindered alignment of the former molecules to the active site of the AChE. Although the indolines with the carbamate in position 6 were also less potent than those with the carbamate in position 4 as BuChE inhibitors, the difference between them was much less than for inhibition of AChE. This observation may be explained by the much wider gorge found in BuChE than in AChE enabling easier access for the compound to the active site. The concentrations of the compounds that inhibited NO and cytokine release from macrophages were at least 4–5 orders of magnitude lower than those inhibiting AChE and BuChE. It is therefore very unlikely that the indoline carbamates will cause any cholinergic adverse effects when administered for the treatment of inflammatory conditions.

Table 2
Inhibitory activity of acetyl and butyrylcholinesterase by indoline carbamates

Compound	IC_{50} AChE (μM)	IC_{50} BuChE (μM)
9	1.3 ± 0.09	0.31 ± 0.03
10	21.3 ± 0.8	2.20 ± 0.03
17	1.8 ± 0.2	1.20 ± 0.04
18	36.0 ± 1.4	4.7 ± 0.2
21	3.7 ± 0.04	0.90 ± 0.08
22	48.0 ± 1.1	4.27 ± 0.18
23	5.6 ± 0.3	0.70 ± 0.13
24	377 ± 10	6.78 ± 0.03
25[a]	7.40 ± 0.03	0.30 ± 0.04
26[a]	55.2 ± 0.1	3.9 ± 0.2
29[a]	0.40 ± 0.04	0.20 ± 0.01

[a] Compounds described previously in Yanovsky et al. (2012).

Summary: This study describes for the first time the anti-inflammatory activity of a series of novel carbamate derivatives of indoline-3- or -1 substituted with propionic esters, primary and tertiary amines in LPS activated macrophages. Several of the indoline derivatives are able to reduce NO, TNF-α and IL-6 proteins at concentrations of 1–10 pM, which are 1000 to 100-fold lower than those of the steroid budesonide. Although the compounds are also AChE inhibitors, their anti-inflammatory activity occurs at much

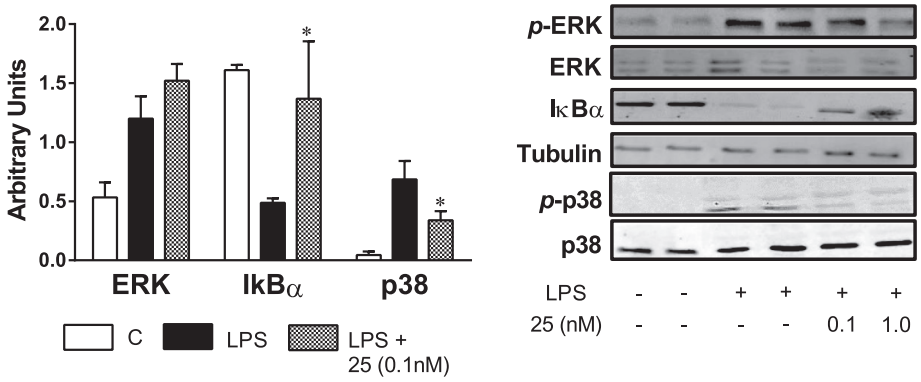


Figure 2. Effect of compound **25** on the phosphorylation of p38 and ERK and IκBα degradation in peritoneal macrophages. Peritoneal macrophages were pre-treated with compound **25** (0.1 nM) for 50 min then activated with LPS (1 μg/mL). Cell lysates were subjected to immunoblot analysis using antibodies against p38, *p*-p38 ERK and *p*-ERK. Each bar represents the mean ± STD from 3–5 measurements. Compound **25** significantly reduced phosphorylation of p38, had no effect on phosphorylation of ERK and significantly reduced the degradation of IκBα. Significantly different from LPS alone $^{*}p$ <0.05.

lower concentrations than those that inhibit this enzyme and is mediated by the reduction in phosphorylation of p38 and increase in IκBα degradation. In addition to their potential anti-inflammatory activity, several of the indoline carbamate esters and amines can prevent cytotoxicity induced by oxidative stress in cardiomyocytes and neuronal cells.[17] This suggests that the compounds may have a beneficial effect in neurodegenerative conditions such as AD and Parkinson's disease in which chronic inflammation and oxidative stress occur, as well as in RA, diabetes type 2 and UC. Further studies are in progress to see whether the compounds can produce beneficial effects in animal models of pathological conditions in which excess activity of cytokines and oxidative–nitrative stress play a leading role.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.03.081.

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