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Aust. J. Chem.
http://dx.doi.org/10.1071/CH16169

Full Paper

Substituted Carbazoles – A New Class of Anthelmintic Agent

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A series of novel carbazoles were synthesized based on structural modifications to lead carbazole **1** ($EC_{100} = 2.5\ \mu M$ against *Haemonchus contortus* in vitro), which was revealed in a small molecule screening program as a potentially promising platform for the development of new anthelmintic drugs. Subsequently, analogues **19**, **21**, **41**, **42** ($EC_{100} = 1.25\ \mu M$, all), and **39** ($EC_{100} = 0.625\ \mu M$) were demonstrated to exhibit enhanced in vitro anthelmintic activity over the lead structure, with compound **39** also being shown to be active in vivo against *Heligmosomoides polygyrus*.

Manuscript received: 18 March 2016.
Manuscript accepted: 28 April 2016.
Published online: 30 May 2016.

Introduction

Parasites are responsible for a variety of diseases detrimental to both human and animal health. In livestock, parasitic infections can lead to substantial decreases in productivity,[1] and in extreme cases animal death. Helminths, in particular gastrointestinal nematodes or roundworms, are generally considered to be the most economically significant parasites affecting sheep and cattle, worldwide.[1] Effective control of these parasites is essential not only for animal welfare, but also in enabling intensive livestock agriculture to produce food and fibre at practical economic cost.

Helminth control relies almost exclusively on the regular application of anti-parasitic agents (e.g. anthelmintics) to the animal in an effort to minimize worm populations and therefore maintain animal performance.[2] The sustained and widespread application of such agents has inadvertently led to the manifestation of organisms impervious to current treatments.[3] Resistance to the three major anthelmintic drug classes, namely, benzimidazoles,[4] imidazothiazoles,[5] and macrocyclic lactones,[6] is now widespread in small ruminants throughout the world,[7] and is likewise becoming an escalating problem in cattle.[8] In terms of new product development, with the exception of the cyclodepsipeptides,[9] represented amongst others by emodepside, and the amino-acetonitrile derivatives or AADs,[10] which include the broad-spectrum anthelmintic monepantel (**2**), no novel anthelmintic class has reached the market in the past 30 years. Despite these advances, history suggests resistance will slowly develop following a few years of use; and in some cases misuse. Accordingly, there is an ongoing need for the development of new anti-parasitic agents, including anthelmintics, to ensure the long-term sustainability of pastoral agriculture.[11,12]

Results and Discussion

Synthesis

A small molecule screening program revealed carbazole **1** (Fig. 1) to display modest, yet encouraging, anthelmintic activity (against *H. contortus)* in vitro. An extended series of carbazoles were synthesized, based on rational modifications to the lead structure, in an endeavour to improve potency.

Compounds **3**–**18** were prepared through the reductive amination of aldehyde **51**, using the appropriate amine in combination with sodium triacetoxyborohydride and acetic acid. Compounds **32** and **33** were synthesized in a similar fashion from carbazoles **52** and **53**, respectively (Scheme 1, Tables 1–3). Rieche formylation[13] of carbazoles **48** and **49**, using dichloromethyl methyl ether and titanium(IV) chloride, led to aldehydes **51** and **52**, respectively. Vilsmeier–Haack formylation[14] of carbazole **50** gave aldehyde **53**. Precursors **48**, **49**, and **50** were accessed through the alkylation of carbazole **47**, using either ethyl bromide, benzyl bromide, or ethyl bromoacetate, respectively. Acid-promoted hydrolysis of ethyl ester **11** revealed carboxylic acid **12**, whereas the amination of carbazole **11** using methylamine gave amide **13**. Oxidative debenzylation[15] of carbazole **32**, using potassium *tert*-butoxide and dimethyl sulfoxide in the presence of oxygen, provided carbazole **31**, with subsequent alkylation, using 2-(dimethylamino) ethyl chloride and sodium hydride, leading to amine **37**. Treatment of ethyl ester **33** with either aqueous hydrochloric acid or

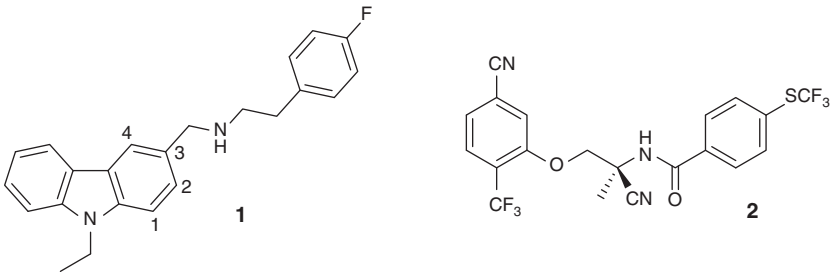
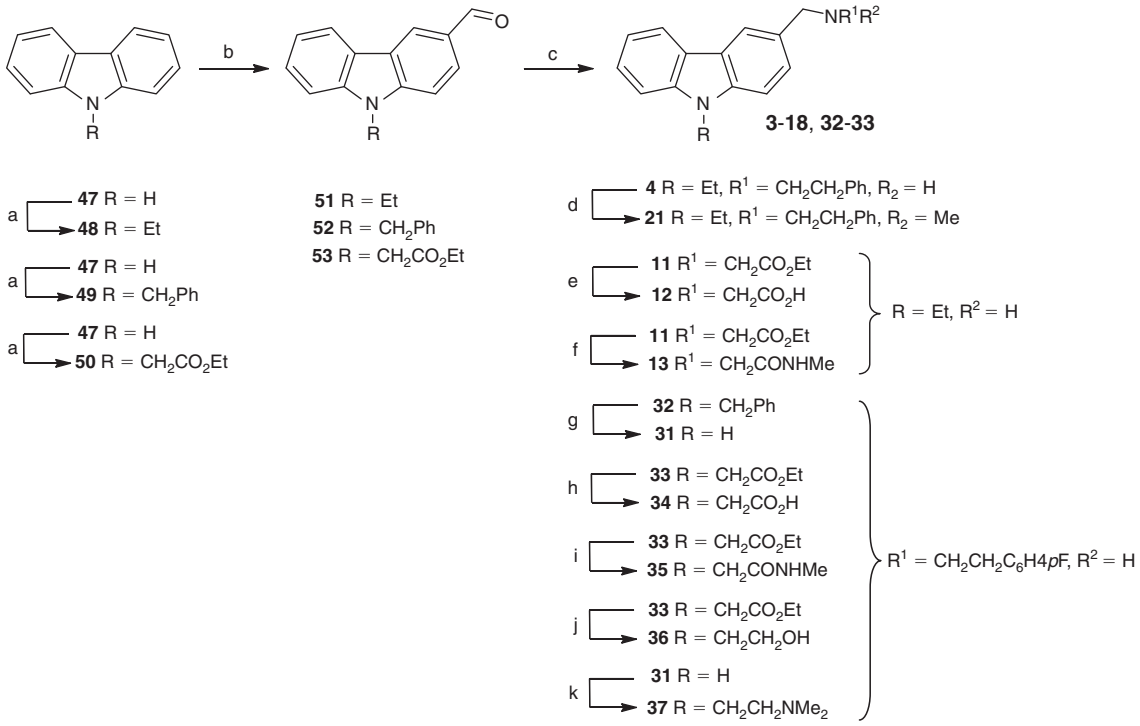


Fig. 1. Structures of lead carbazole **1** and monepantel (**2**).



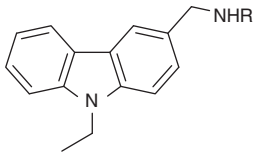
Scheme 1. Reagents and conditions: (a) NaH, EtBr, DMF, rt, 18 h, 76 % (to give **48**) or NaH, $PhCH_2Br$, DMF, rt, 18 h, 75 % (to give **49**) or NaH, $BrCH_2CO_2Et$, DMF, rt, 18 h, 60 % (to give **50**); (b) **48**, Cl_2CHOMe, $TiCl_4$, DCM, −78°C, 4 h, 89 % (to give **51**) or **49**, Cl_2CHOMe, $TiCl_4$, DCM, −78°C, 4 h, 83 % (to give **52**) or **50**, $POCl_3$, DMF, 100°C, 2 h, 55 % (to give **53**); (c) RNH_2, $NaBH(OAc)_3$, AcOH, THF, rt, 18 h (**3**–**18** and **32**–**33**, see Tables 1, 2 and 3); (d) **4**, aq. formaldehyde, $NaBH(OAc)_3$, AcOH, THF, rt, 18 h, 75 %; (e) 6 M HCl(aq), reflux, 10 min, 55 %; (f) $MeNH_2$, EtOH, rt, 18 h, 89 %; (g) *t*-BuOK, O_2, THF-DMSO (4 : 1), rt, 1 h, 69 %; (h) 6 M HCl(aq), reflux, 10 min, 60 %; (i) $MeNH_2$, EtOH, rt, 18 h, 84 %; (j) $LiAlH_4$, THF, rt, 18 h, 77 %; (k) NaH, $ClCH_2CH_2NMe_2$.HCl, DMF, rt, 18 h, 70 %.

ethanolic methylamine afforded carboxylic acid **34** and amide **35**, respectively. Reduction of ester **33** using lithium aluminium hydride led to alcohol **36**. Reductive amination of carbazole **4** using aqueous formaldehyde gave tertiary amine derivative **21**.

Compounds **19**, **22**–**26**, **29**, and **30** were prepared from common aldehyde precursor **51** (Scheme 2). Treatment of aldehyde **51** with hydroxylamine led to oxime **56**, with subsequent *O*-benzylation affording carbazole **25**. Reduction of oxime **56** using lithium aluminium hydride gave amine **57**, which was then acylated to reveal amide **22**. Alternatively, reduction of aldehyde **51** using sodium borohydride afforded alcohol **60**, which was then alkylated using benzyl bromide to give ether **26**. Nitrile **59**, prepared over two-steps via oxime **56**, was converted into amidine **24** through reaction with phenethylamine under Pinner-type conditions. Oxidation of aldehyde **51** using potassium permanganate led to carboxylic acid **58**, which was then converted into amide **23** via the corresponding acid chloride. β-Nitrovinyl derivative **54** was accessed through the Henry reaction of nitromethane and aldehyde **51**, with lithium aluminium hydride induced reduction leading to amine **55**; which then underwent reductive amination in the presence of benzaldehyde to afford carbazole **19**. Carbazole **29** was prepared over two-steps from aldehyde **51** using Wittig chemistry (to reveal alkene **30**), followed by palladium(0)-catalyzed hydrogenation.

Compounds **20**, **27**, and **28** were accessed from common aniline precursor **62**, which was prepared via the sequential nitration and reduction of carbazole **48** (Scheme 3). Reductive amination of aniline **62** with hydrocinnamaldehyde, under conditions similar to those described previously, gave carbazole **20**. Acylation of aniline **62** with cinnamoyl chloride afforded cinnamide **28**, which was subsequently hydrogenated over palladium(0) to reveal saturated amide **27**.

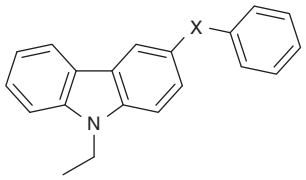
Table 1. Anthelmintic activity of compounds 3–16



Compound	R	Yield [%]	Log P^A	$EC_{100}{}^B$ [μM]
3	$CH_2CH_2C_6H_4pCl$	65	5.34	2.5
4	CH_2CH_2Ph	64	4.78	2.5
5	2-Pyridinylmethyl	64	3.73	5
6	2-Furanylmethyl	69	3.26	2.5
7	2-Thienylmethyl	71	4.62	5
8	$CH_2CH_2CMe_3$	73	4.81	1.25
9	CH_2CH_2OH	70	2.25	10
10	CH_2CH_2OMe	39	2.61	10
11	CH_2CO_2Et	73	2.78	>10
12	CH_2CO_2H	–	2.17	>10
13	$CH_2CONHMe$	–	1.76	>10
14	CH_2CH_2NHAc	62	1.80	>10
15	$CH_2CH_2NMe_2$	65	2.77	5
16	CH_2CH_3	68	3.10	5
1	–	–	4.94	2.5
Monepantel	–	–	3.00	0.0125

[A]Log *P* (*in silico*, *ChemBioDraw Ultra version 12*).
[B]Against *H. contortus* in vitro.

Table 2. Anthelmintic activity of compounds 17–30

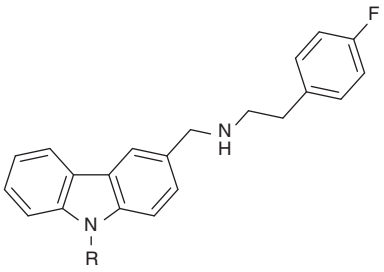


Compound	X	Yield [%]	Log P^A	$EC_{100}{}^B$ [μM]
17	CH_2NHCH_2	68	4.64	2.5
18	$CH_2NHCH_2CH_2CH_2$	64	5.20	2.5
19	$CH_2CH_2NHCH_2$	–	4.78	1.25
20	$NHCH_2CH_2CH_2$	–	5.13	>10
21	$CH_2N(Me)CH_2CH_2$	–	5.16	1.25
22	$CH_2NHC(=O)CH_2$	–	4.02	>10
23	$C(=O)NHCH_2CH_2$	–	4.36	>10
24	$C(=NH)NHCH_2CH_2$	–	4.88	>10
25	$C{=}NOCH_2$	–	5.33	>10
26	CH_2OCH_2	–	4.72	>10
27	$NHC(=O)CH_2CH_2$	–	4.37	>10
28	NHC(=O)CH=CH	–	4.35	>10
29	$CH_2CH_2CH_2CH_2$	–	6.55	>10
30	$CH{=}CHCH_2CH_2$	–	6.23	>10
1	–	–	4.94	2.5
Monepantel	–	–	3.00	0.0125

[A]Log *P* (*in silico*, *ChemBioDraw Ultra version 12*).
[B]Against *H. contortus* in vitro.

Compound **38**, a regioisomer of lead carbazole **1**, was prepared over 8 steps from commercially available 2-anthranillic acid methyl ester (**63**); using a modified Fischer–Borsche approach[16] (Scheme 4). Diazotization of aniline **63**, followed by tin(II) chloride induced reduction, led to hydrazine **64**, which was then reacted with cyclohexanone in the presence of acetic acid[17] to afford tetrahydrocarbazole **65**. Catalytic dehydrogenation[17] of carbazole **65**, using palladium(0) at elevated temperature, gave carbazole **66**. Methyl ester **66** was reduced using lithium aluminium hydride to give alcohol **67**, which was then oxidized using pyridinium chlorochromate to reveal aldehyde **68**. Alkylation of carbazole **68** using ethyl bromide gave carbazole **69**. Finally, reductive amination of aldehyde **69** and 2-(*p*-fluorophenyl)ethylamine, in the presence of triacetoxyborohydride and acetic acid, led to target carbazole **38**.

Table 3. Anthelmintic activity of analogues 31–37



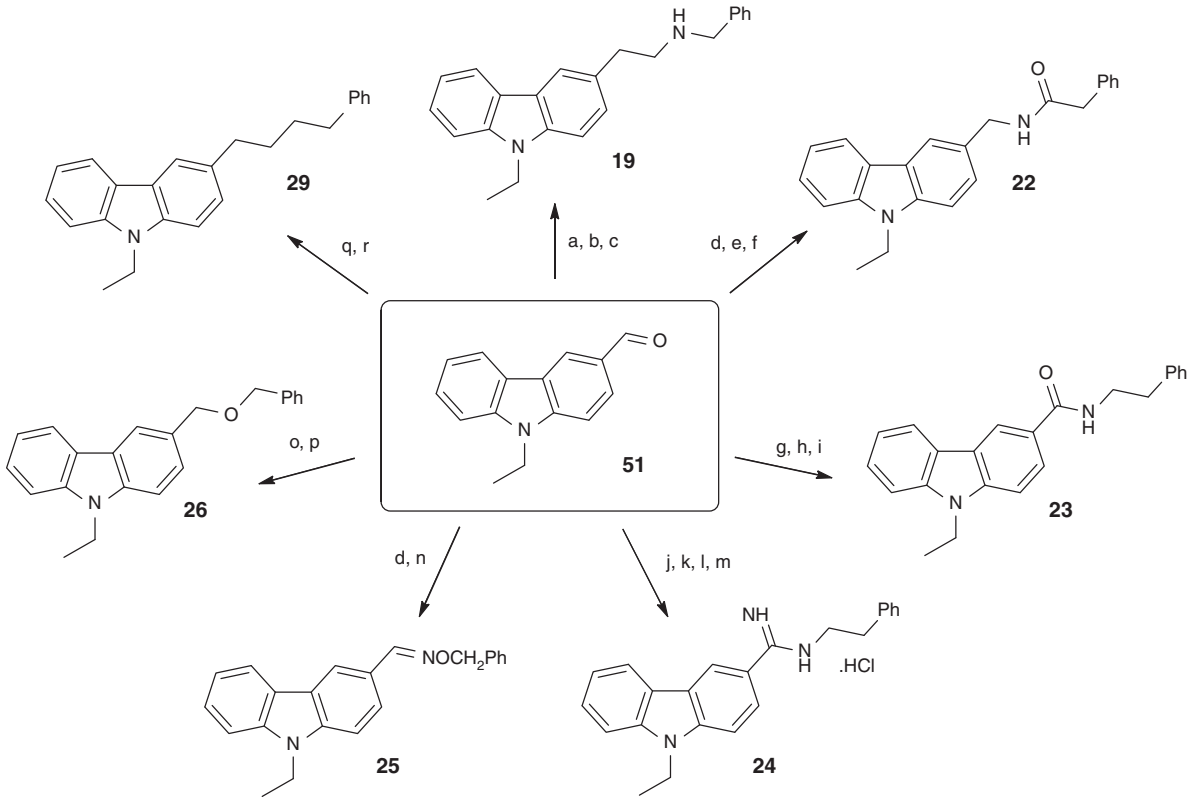
Compound	R	Yield [%]	Log P^A	$EC_{100}{}^B$ [μM]
31	H	–	4.36	2.5
32	CH_2Ph	75	6.33	10
33	CH_2CO_2Et	69	4.47	10
34	CH_2CO_2H	–	3.87	5
35	$CH_2CONHMe$	–	3.45	5
36	CH_2CH_2OH	–	4.08	2.5[b]
37	$CH_2CH_2NMe_2$	–	4.60	2.5
1	–	–	4.94	2.5
Monepantel	–	–	3.00	0.0125

[A]Log *P* (*in silico*, *ChemBioDraw Ultra version 12*).
[B]Against *H. contortus* in vitro.

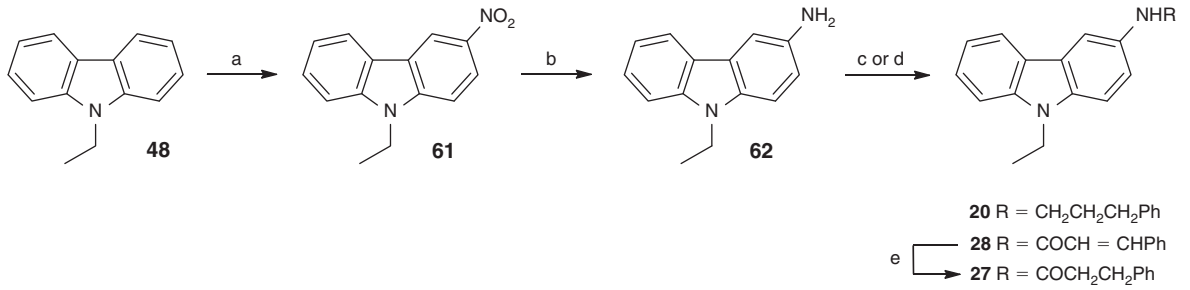
Compound **39**, a second regioisomeric example of carbazole **1**, was built from commercially available 4-chloro-3-nitrobenzaldehyde (**70**) over 4 steps; via a modified Cadogan cyclization[16,18] (Scheme 5). Suzuki coupling[19] of phenylboronic acid and aldehyde **70**, in the presence of tetrakis (triphenylphosphine)palladium(0), led to 2-nitrobiphenyl derivative **71**. Cyclization of compound **71** was achieved via a reductive deoxygenation mechanism,[20] using triphenylphosphine at elevated temperature, to give carbazole **72**. Alkylation of carbazole **72** furnished compound **73**. Reductive amination of **73** with 2-(*p*-fluorophenyl)ethylamine, as described for carbazole **38**, afforded carbazole **39**.

In similar fashion, compound **40**, a third regioisomeric analogue of carbazole **1**, was prepared over 5 steps from commercially available 1-bromo-2-nitrobenzene (**74**) (Scheme 6). Sequential Suzuki coupling[21] of 2-methylphenylboronic acid and aryl bromide **74** (to give **75**), followed by reductive cyclization,[20] gave carbazole **76**. Oxidation of compound **76** using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) under UV irradiation revealed aldehyde **77**, with subsequent ethylation leading to carbazole **78**. Finally, reductive amination of aldehyde **78** with 2-(*p*-fluorophenyl)ethylamine, as detailed previously, furnished carbazole **40**.

Compounds **41**–**43** were prepared from common aldehyde precursor **51** over 2 steps (Scheme 7). Reaction of carbazole **51**



Scheme 2. Reagents and conditions: (a) $MeNO_2$, NH_4OAc, AcOH, 80°C, 2 h (to give **54**); (b) $LiAlH_4$, THF, rt, 18 h, 61 % (over 2 steps to give **55**); (c) PhCHO, $NaH(OAc)_3$, AcOH, THF, rt, 18 h, 67 %; (d) $NH_2OH.HCl$, NaOH(aq), EtOH, rt, 18 h, 83 % (to give **56**); (e) $LiAlH_4$, THF, rt, 18 h, 60 % (to give **57**); (f) $PhCH_2COCl$, Et_3N, DMAP, DCM, rt, 18 h, 82 %; (g) $KMnO_4$, acetone, rt, 3 h, 86 % (to give **58**); (h) $SOCl_2$, reflux, 3 h; (i) $PhCH_2CH_2NH_2$, Et_3N, DMAP, DCM, rt, 18 h, 82 % (over 2 steps); (j) $NH_2OH.HCl$, Py, rt, 18 h; (k) Ac_2O, reflux, 6 h, 61 % (to give **59** over 2 steps); (l) 4 M HCl/1,4-dioxane, EtOH, rt, 48 h; (m) $PhCH_2CH_2NH_2$, EtOH, reflux, 3 days, 15 % (over 2 steps); (n) NaH, $PhCH_2Br$, DMF, rt, 18 h, 86 %; (o) $NaBH_4$, EtOH-THF (1 : 1), rt, 3 h, 96 % (to give **60**); (p) NaH, $PhCH_2Br$, DMF, rt, 18 h, 59 %; (q) $PhCH_2CH_2CH_2PPh_3Br$, *n*-BuLi, THF, −78°C to rt, 2 h, 55 % (to give **30**); (r) H_2, Pd/C, EtOAc, rt, 18 h, 100 %.



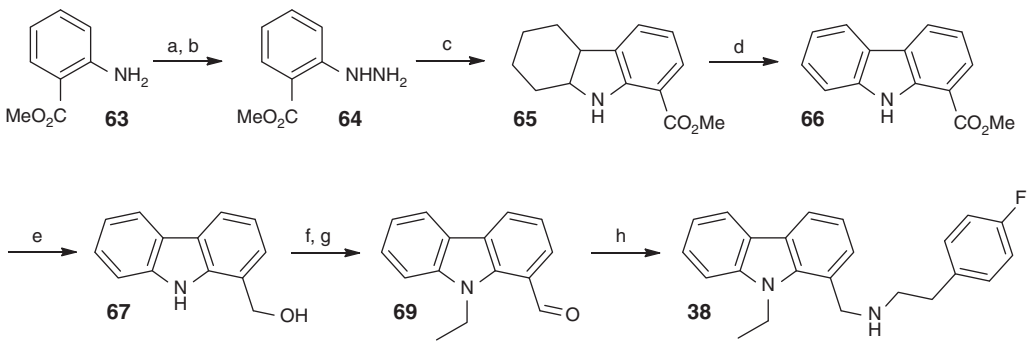
Scheme 3. Reagents and conditions: (a) HNO_3(aq), $ClCH_2CH_2Cl$, 10°C, 1 h, 81 %; (b) $SnCl_2{\cdot}2H_2O$, EtOH, reflux, 18 h, 86 %; (c) $PhCH_2CH_2CHO$, $NaH(OAc)_3$, AcOH, THF, rt, 18 h, 50 % (to give **20**) or (d) PhCH=CHCOCl, Et_3N, DMAP, DCM, rt, 18 h, 88 % (to give **28**); (e) H_2, Pd/C, EtOAc, rt, 18 h, 86 %.

with the appropriate lithiated pyridine, generated from the corresponding bromopyridine using *n*-butyllithium, gave pyridinylmethanols **79**–**81**. Reduction of compounds **79**–**81**, using platinum(IV) oxide in the presence of hydrochloric acid, led directly to dehydroxylated piperidines **41**–**43**; whereas performing the same reaction in glacial acid afforded piperidinylmethanols **44**–**46**.

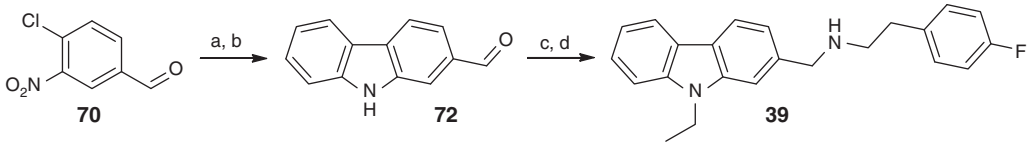
Biological Evaluation

Compounds **3**–**46** were screened in vitro for activity against the helminth *Haemonchus contortus*; where lead carbazole **1** was previously revealed to have an EC_{100} of 2.5 μM (the EC_{100} of a compound being the concentration at which 100% of the nematodes present were killed) (Tables 1–5). To elucidate which regions of the lead structure were potentially involved in binding to the alleged macromolecular target, a comprehensive structure–activity study was undertaken. In a rational, stepwise fashion, each functional group was now modified, masked, or removed to determine its importance/contribution towards biological activity.

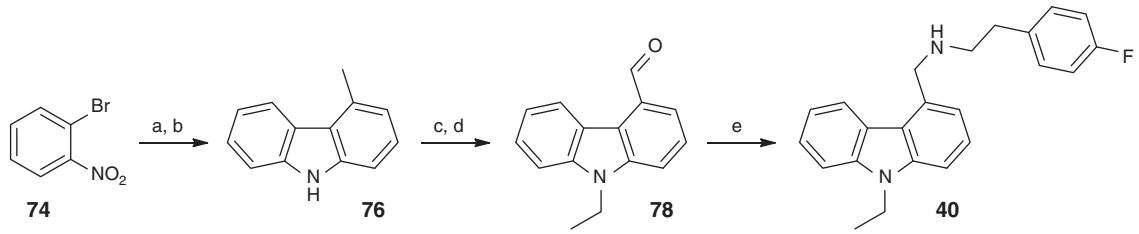
Lipophilic substituents have the capacity to interact with hydrophobic regions of a binding domain in several different ways. Although recognised as being weaker than both hydrogen bonds and ion-pair interactions, given that such moieties can



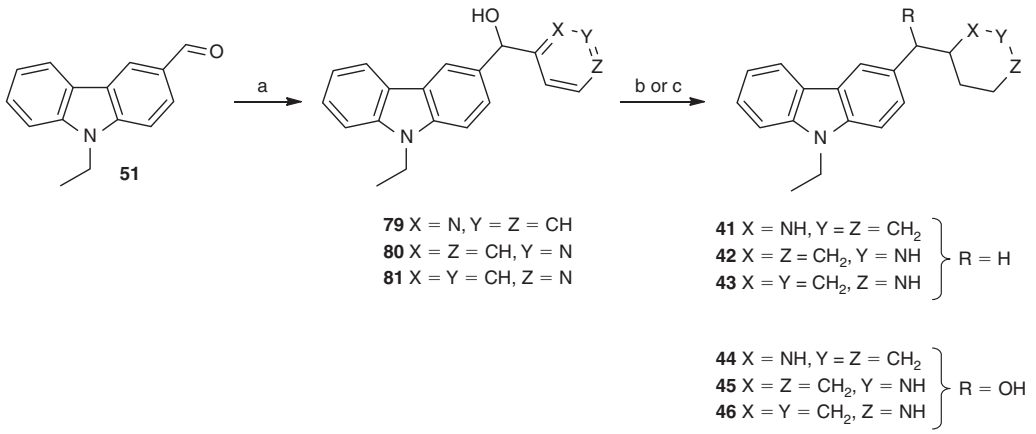
Scheme 4. Reagents and conditions: (a) $NaNO_2$, HCl(aq), H_2O, <10°C, 0.5 h; (b) $SnCl_2{\cdot}H_2O$, HCl(aq), H_2O; (c) cyclohexanone, AcOH, reflux, 3 h, 40 % (over 3 steps); (d) Pd/C, 260°C, 2 h; (e) $LiAlH_4$, THF, rt, 1 h, 56 % (over 2 steps); (f) PCC, DCM, rt, 1.5 h, 76 % (to give **68**); (g) NaH, EtBr, DMF, rt, 18 h, 90 %; (h) *p*-$FC_6H_4CH_2CH_2NH_2$, $NaBH(OAc)_3$, AcOH, THF, rt, 18 h, 70 %.



Scheme 5. Reagents and conditions: (a) $PhB(OH)_2$, $Pd(Ph_3P)_4$, K_2CO_3, PhMe, 100°C, 18 h (to give **71**); (b) Ph_3P, DMA, 150°C, 18 h, 71 % (over 2 steps); (c) NaH, EtBr, DMF, rt, 18 h, 89 % (to give **73**); (d) *p*-$FC_6H_4CH_2CH_2NH_2$, $NaBH(OAc)_3$, AcOH, THF, rt, 18 h, 70 %.

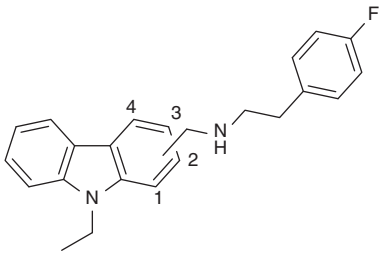


Scheme 6. Reagents and conditions: (a) 2-Methylphenylboronic acid, $Pd(Ph_3P)_4$, Na_2CO_3, DME-EtOH, reflux, 18 h, 94 % (to give **75**); (b) Ph_3P, DMA, 150°C, 18 h, 91 %; (c) DDQ, 1,4-dioxane-H_2O, AcOH, 110°C, μν (150 W), 5.5 h, 21 % (to give **77**); (d) NaH, EtBr, DMF, rt, 18 h, 93 %; (e) *p*-$FC_6H_4CH_2CH_2NH_2$, $MgSO_4$, DCM, rt, 18 h then AcOH, 4Å MS, 50°C, 16 h then $NaBH_4$, MeOH, rt, 18 h, 63 %.



Scheme 7. Reagents and conditions: (a) 2-bromopyridine, *n*-BuLi, Et_2O, −78°C, 4 h, 84 % (to give **79**) or 3-bromopyridine, *n*-BuLi, THF, −78°C, 4 h, 69 % (to give **80**) or 4-bromopyridine (prepared from 4-bromopyridine hydrochloride and K_2CO_3), *n*-BuLi, THF, −78°C, 4 h, 64 % (to give **81**); (b) **79**, H_2, PtO_2, HCl(aq), EtOH, 18 h, 100 % (to give **41**) or **80**, H_2, PtO_2, HCl(aq), EtOH, 18 h, 91 % (to give **42**) or **81**, H_2, PtO_2, HCl(aq), EtOH, 18 h, 90 % (to give **43**); (c) **79**, H_2, PtO_2, AcOH, 18 h, 51 % (to give **44**) or **80**, H_2, PtO_2, AcOH, 18 h, 64 % (to give **45**) or **81**, H_2, PtO_2, AcOH, 18 h, 67 % (to give **46**).

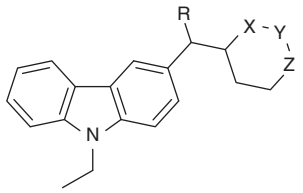
Table 4. Anthelmintic activity of analogues 38–40



Compound	Ring substituent position	Log P^A	$EC_{100}{}^B$ [μM]
38	1	4.94	10
39	2	4.94	0.62[C]
40	4	4.94	1.25
1	3	4.94	2.5
Monepantel	–	3.00	0.0125

[A]Log *P* (*in silico*, *ChemBioDraw Ultra version 12*).
[B]Against *H. contortus* in vitro.
[C]Lowest dose tested.

Table 5. Anthelmintic activity of analogues 41–46



Compound	R	X	Y	Z	Log P^A	$EC_{100}{}^B$ [μM]
41	H	NH	CH_2	CH_2	3.76	1.25
42	H	CH_2	NH	CH_2	3.77	1.25
43	H	CH_2	CH_2	NH	3.70	2.5
44	OH	NH	CH_2	CH_2	2.94	10
45	OH	CH_2	NH	CH_2	2.73	>10
46	OH	CH_2	CH_2	NH	2.68	>10
1	–	–	–	–	4.94	2.5
Monepantel	–	–	–	–	3.00	0.0125

[A]Log *P* (*in silico*, *ChemBioDraw Ultra version 12*).
[B]Against *H. contortus* in vitro.

often be present in sizeable numbers, the resulting cumulative effect can contribute significant binding. Aryl–aryl interactions are of particular importance, for instance the interactions between aromatic ligands and aryl-containing amino acids such as phenylalanine, tyrosine, tryptophan, and histidine.[22] The introduction of substituents or insertion of heteroatoms into aromatic rings can have a pronounced influence on both the strength and geometry of aryl–aryl interactions. As a rule, the stacking arrangements of electron-rich and electron-poor aromatic rings profit from charge transfer. In similar fashion, stacking between electron-deficient rings is generally preferred over the stacking of electron-rich ones.[23] To probe the importance of the aryl ring substituent on the activity of lead carbazole **1**, chlorophenyl derivative **3** was prepared and subsequently demonstrated to be equipotent to its fluorinated counterpart. Unsubstituted carbazole **4** was likewise shown to be of similar activity to halogenated carbazoles **1** and **3**. It is known that the insertion of a heteroatom into an aryl ring produces an electron-deficient system, which in turn can improve aryl–aryl stacking interactions with aromatic amino acid side-chains. Of the heteroaromatic derivatives prepared, furan **6** was revealed to be comparable to carbazole **1**, whereas pyridine **5** and thiophene **7** were found to be marginally less active; linker shortened analogues **6**–**8** were prepared on synthetic grounds (and can be directly compared with carbazole **17**). As an example of a non-aromatic hydrophobic substituent (hence removing the capacity of the side-chain group to form π–π interactions), *tert*-butyl derivative **8** was demonstrated to be of similar potency to its aryl counterparts. With regards to the overall lipophilicity of compounds **3**–**8**, as determined by their *in silico* generated log *P* values, no apparent trends were observed in this particular instance (Table 1).

Next, a selection of carbazoles bearing polar functional groups were investigated. Hydrogen bonds are acknowledged as being one of the most important specific interactions in the biological recognition process. Among others, alcohols, ketones, amides, ethers, and esters can all act as hydrogen bond acceptors, and in the case of alcohols and amides, hydrogen bond donors. While carboxylic acids are less common functional groups in both lead compounds and drugs, they are still relatively prevalent. Again, they have the capacity to form hydrogen bonds, either as the free acid or as the ionized carboxylate ion. In addition, carboxylate ions can form strong ionic interactions with charged aminium and guanidinium ions (e.g. lysine and arginine residues, respectively). Alcohol **9** was subsequently shown to be significantly less active than carbazole **1**, as were methyl ether **10**, ethyl ester **11**, carboxylic acid **12**, and amide regioisomers **13** and **14**. On the basis of these findings it would appear that no significant hydrogen bonding interaction was attained through the incorporation of these specific polar moieties into this particular region of the molecule. This is not to say that the putative binding pocket does not possess the capacity to form hydrogen bonds, but that the intermolecular distances, geometry, and directionality involved may not have been optimal to allow for a favourable interaction. With regards to overall lipophilicity, a potential correlation between the activity of compounds **9**–**14** and their lower log *P* values could also be put forward as a further reason for their inferior performance in vitro (compared with their more lipophilic counterparts, compounds **3**–**8**, Table 1).

Amines also have the potential to form hydrogen bonds, the specific nature of which is largely determined by whether the amine is in an ionized or non-ionized form; which itself is dictated by the pH of the physiological environment. When ionized, the capacity of an amine to operate as a hydrogen bond acceptor is lost; however, on the other hand, protonated amines can now act as much stronger hydrogen bond donors. Moreover, aminium ions have the potential to form strong ionic interactions with charged carboxylate-containing amino acids such as aspartate and glutamate. A subtle recovery in activity was noted for amine **15** (versus other examples bearing polar functional groups within the series, namely compounds **9**–**14**). π-Cation interactions are also known to play an important role in the binding of aminium groups to aromatic or heteroaromatic moieties.[24] It is recognised that methyl groups interact favourably with the π-face of aromatic rings when bound to an electronegative atom. Given that a positively charged nitrogen is a particularly strong electron-withdrawing species, the

interaction between alkylammonium ions and aromatic rings can lead to even stronger alkyl–aryl interactions. Carbazole **16**, an example bearing no terminal group, only the linker, was found to be of moderate activity.

In parallel to the preparation of compounds **3**–**16**, carbazoles **17** and **18** were designed to probe the importance of linker length on anthelmintic activity (Table 2). Homologues **17** and **18**, incorporating linkers of 3 and 5 atoms, respectively (while retaining the position of the linker nitrogen), were demonstrated to be of similar activity to carbazole **4** (4 atom linker example). This would appear to suggest that there is a degree of tolerance in terms of the postulated binding of the linker amine in combination with that of the carbazole core and the side-chain aromatic moiety. With regards to carbazole **17**, as a by-product of removing a methylene unit from the side-chain, the basicity of the amine was concurrently lowered due to the increasing influence of the neighbouring electron-withdrawing phenyl group (based on *in silico* pK_a values of 8.7 and 9.3 for compounds **17** and **1**, respectively; *ACD laboratories version 12*). For compounds **1**, **4**, and **17**–**19** it should be noted that at pH 7.4 and below the proportion of ionized amine is calculated to be $> 95\,\%$; thus the linker amine has the capacity to act as both a hydrogen bond donor and/or form strong ion-pair interactions.

Next, the position of the nitrogen atom within the linker was investigated through the preparation of carbazoles **19** and **20**. Moving the linker nitrogen one atom further away from the carbazole core, as found in analogue **19**, led to an improvement in activity, suggesting that a more favourable interaction was attained as a consequence. Conversely, carbazole **20** was found to be inactive, most likely due to the amine character of the molecule being removed to reveal a non-basic aniline derivative. Gratifyingly, tertiary amine **21** was demonstrated to be more potent than its secondary amine counterpart, carbazole **4**. Potential explanations for this observation may relate to those outlined earlier for compound **15**. Replacing the amine linker of carbazole **4** with an amide, through regioisomers **22** and **23**, again likely reiterated the importance of having a basic group in this region of the molecule. This was possibly further reinforced by the fact that both oxime **25** and ether **26** were similarly devoid of all activity. Curiously amidine **24**, prepared as a linker example incorporating a non-amine basic moiety, was demonstrated to be inactive. Finally, carbazoles **29** and **30**, possessing linkers solely constituted of carbon atoms, were also confirmed to be inactive, as were cinnamides **27** and **28**.

Derivatization of the carbazole ring nitrogen subsequently revealed both benzyl and ethoxycarbonylmethyl substituted analogues, compounds **32** and **33** respectively, to be weakly active. A slight recovery in activity was noted for carboxylic acid derivative **34** and amide **35**, while alcohol **36** and amine **37**, along with unsubstituted carbazole **31**, were found to be equipotent to lead carbazole **1** (Table 3). In the absence of any clear trend in terms of molecular interaction (other than possibly steric intolerance), the overall lipophilicity of the molecule may be the dominant parameter in this instance; it could be postulated that the optimal log *P* within this series of compounds is between 4–5 (based on *in silico* log *P* values; *ChemBioDraw Ultra version 12*).

Next, through the preparation of regioisomeric carbazoles **38**–**40**, our focus moved onto determining the importance of the location of the side-chain (Table 4). Incorporation of the substituent at the 1-position of the carbazole ring resulted in a marked loss in activity. Conversely, analogues **39** and **40** were revealed to be of greater anthelmintic activity than the lead structure. It could be postulated that the spatial orientation of both the linker amine and carbazole core of carbazoles **1**, **39**, and **40** is more favourable, in terms of binding to the target, than it is in example **38**.

Table 6. In vivo evaluation of compounds 1 and 39

Compound	Concentration [μM]	Average adult nematodes[A]
1	100	39.8 ± 4.0
39	100	14.9 ± 4.4
DMSO	Control	32.4 ± 5.5

[A]Against *H. polygyrus*.

In a final effort to further optimize the spatial positioning of the linker amine with respect to the carbazole core, piperidine derivatives **41**–**43** were prepared (Table 5). Gratifyingly, compounds **41** and **42** were demonstrated to be more active than carbazole **1**, while compound **43** was found to be equipotent. Precursors **44**–**46** were revealed to be inactive, suggesting that the presence of a hydroxy group in this position led to an unfavourable interaction with the binding domain.

Under these assay conditions, the in vitro EC_{100} for monepantel (**2**), the active ingredient in Zolvix, a commercially available broad spectrum anthelmintic for the treatment of gastrointestinal roundworms in sheep, was found to be 0.0125 ppm (against *H. contortus*).

Based on their performance in vitro, compounds **1** and **39** were next screened for anthelmintic activity against the helminth *H. polygyrus*, in mice (Table 6). Whereas lead carbazole **1** was demonstrated to be inactive in vivo, encouragingly, compound **39** was found to have an EC_{50} in the region of 50 μM.

Conclusion

In summary, carbazole **1** underwent an array of structural modifications to reveal compound **39**, which exhibited a minimum 4-fold improvement in anthelmintic activity (in vitro) over the lead structure. Although monepantel (**2**) was shown to be around 50 times more potent against *H. contortus* in vitro, the fact that compound **39** displayed activity in vivo (against *H. polygyrus*) suggests that carbazoles remain a genuine platform for further development in the search for a new class of anthelmintic agent.

Experimental

Synthesis

General Experimental Methods

All reagents were used as supplied unless stated otherwise. Analytical thin-layer chromatography (TLC) was carried out on pre-coated silica gel plates (Merck/UV_{254}) and products were visualized by UV fluorescence and/or staining. Flash chromatography was performed using silica gel (Riedel-de Haën, particle size 0.032–0.063 mm). NMR spectra were recorded on a Bruker AVANCE 300 (^{1}H, 300 MHz) spectrometer at 298 K. For ^{1}H NMR data, chemical shifts are described in parts per million (ppm) relative to tetramethylsilane (δ 0.00) and are reported consecutively as position (δ_H), relative integral, multiplicity (s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant (*J*, Hz), and assignment. Mass spectra were recorded on a VG-70SE mass spectrometer (EI, CI, and FAB). The purity of all target compounds was assigned using reverse phase-HPLC

(Dionex P680 system using a Phenomenex Gemini C_{18}-Si column, 150 mm × 4.6 mm, 5 μm) – eluted using a gradient of 100 : 0 % A/B to 5 : 95 % A/B over 15 min at 1 mL min^{-1}; where solvent A was water (+0.1 % trifluoroacetic acid) and solvent B was CH_3CN (+0.1 % trifluoroacetic acid); with detection at 210, 254, and 280 nm.

*9-Ethyl-*N*-[2-(4-fluorophenyl)ethyl)-9*H*-carbazole-2-methanamine (**39**)*

A solution of **73** (160 mg, 0.72 mmol), 2-(4-fluorophenyl) ethylamine (100 mg, 0.72 mmol), and glacial acetic acid (1 drop, cat.) in anhydrous tetrahydrofuran (5 mL) was stirred at room temperature for 1 h. Sodium triacetoxyborohydride (150 mg, 0.72 mmol) was added in a single portion and the mixture stirred for a further 18 h. The reaction mixture was diluted with ethyl acetate and washed with a saturated aqueous solution of sodium hydrogen carbonate. The combined organic phases were washed with water, and then brine, dried over anhydrous magnesium sulfate, and the solvent removed under vacuum. Purification by column chromatography (ethyl acetate) afforded **39** as a pale orange oil (175 mg, 0.51 mmol, 70 %). δ_H (300 MHz, $CDCl_3$) 1.38 (3H, t, *J* 7.2, NCH_2CH_3), 2.80 (2H, m), 2.94 (2H, m), 3.98 (2H, s, CH_2NH), 4.31 (2H, q, *J* 7.2, NCH_2CH_3), 6.91–6.98 (2H, m, ArH), 7.09–7.22 (4H, m, ArH), 7.31–7.45 (3H, m, ArH), 8.01 (1H, d, *J* 7.8, ArH), 8.05 (1H, d, *J* 7.8, ArH). δ_C (75 MHz, $CDCl_3$) 13.7 (CH_3), 35.5 (CH_2), 37.4 (CH_2), 50.5 (CH_2), 54.5 (CH_2), 107.7 (CH), 108.4 (CH), 115.1 (CH, d, J_{CF} 20.3), 118.7 (CH), 119.1 (CH), 120.1 (CH), 120.2 (CH), 121.9 (C), 122.8 (C), 125.3 (CH), 130.0 (CH, d, J_{CF} 7.5), 135.7 (C, d, J_{CF} 3.0), 138.0 (C), 140.1 (C), 140.2 (C), 161.4 (C, d, J_{CF} 242). *m/z* (ESI) 347 ($[M + H]^+$, 90 %), 208 (100).

*9-Ethyl-9*H*-carbazole-2-carboxaldehyde (**73**)*[25]

To a solution of **72** (0.51 g, 2.61 mmol) in anhydrous dimethylformamide (10 mL) at 0°C under nitrogen was carefully added sodium hydride (130 mg, 3.26 mmol, 60 % w/w in oil) in a single portion. After 0.5 h ethyl bromide (0.22 mL, 2.87 mmol) was added and the mixture was stirred at room temperature overnight. The reaction mixture was poured onto ice and extracted with ethyl acetate. The combined organic phases were washed with water, and then brine, dried over anhydrous magnesium sulfate, and the solvent removed under vacuum. Purification by column chromatography (hexane/ethyl acetate, 20 : 1) afforded **73** as a pale yellow solid (0.52 g, 2.33 mmol, 89 %). Spectroscopic data was in agreement with that reported in the literature.[25]

*9*H*-Carbazole-2-carboxaldehyde (**72**)*[26]

A solution of **71** (2.5 g) and triphenylphosphine (14.2 g, 54 mmol) in dimethylacetamide (20 mL) was heated at 150°C overnight. The reaction was allowed to cool, diluted with ethyl acetate, washed with water, and then brine, dried over anhydrous magnesium sulfate, and the solvent removed under vacuum. Purification by column chromatography (hexane/ethyl acetate, 7 : 1 then 3 : 1) afforded **72** as a light brown solid (1.50 g, 7.7 mmol, 71 % over 2 steps). Spectroscopic data was in agreement with that reported in the literature.[26]

*4-Formyl-2-nitrobiphenyl (**71**)*[19]

A solution of 4-chloro-3-nitrobenzaldehyde (**70**) (2.0 g, 10.8 mmol), phenylboronic acid (1.43 g, 11.8 mmol), and aqueous potassium carbonate (11 mL, 21.6 mmol, 2 M) in toluene (15 mL) was purged with nitrogen, followed by the addition of tetrakis(triphenylphosphine)palladium(0) (125 mg, 0.11 mmol). The mixture was heated at 100°C overnight. The reaction was allowed to cool, diluted with diethyl ether, and washed with water, and then brine, dried over anhydrous magnesium sulfate, and the solvent removed under vacuum to afford **71** as a pale yellow solid (2.5 g), which was used without further purification. Spectroscopic data was in agreement with that reported in the literature.[19]

Biological Evaluation

Compounds (**3**–**46**) were screened for anti-parasitic activity against the helminth *Haemonchus contortus* using an EC_{100} assay (the EC_{100} of a compound being the concentration at which 100% of the nematodes present were killed). *Haemonchus contortus* were recovered from faeces using literature methods.[27]

A centrifuge tube containing eggs was shaken well and a 100 μL aliquot of the egg solution taken; the eggs were counted using the McMaster Chamber in accordance with the manufacturer's instructions. Distilled water was either added or removed (by centrifuging and removing the appropriate quantity of water) to obtain an egg solution with a concentration of 100 eggs per 100 μL. Working stock solutions of each compound (100 μM) were prepared by dissolving and/or diluting each compound in DMSO. Additional dilutions were performed with DMSO, as required.

The compounds were assayed using 96 well Nunc tissue culture plates. Agar (Merck-101614) was prepared as a 2 % solution and then heated by microwave before cooling to ~45°C. A phosphate buffered saline (PBS, 0.85 %) solution was prepared by dissolving one PBS tablet (Sigma P4417) in 125 mL of distilled water. Earle's balanced salt solution (1X) was prepared from 10X Earle's balanced salt solution (Sigma E7510). A yeast solution (1 %) was prepared from 0.25 g of yeast extract (Sigma Y-1000), 22.5 mL of 0.85 % PBS solution, and 2.5 mL of Earle's balanced salt solution (1X).

Larval development assay (LDA) media was prepared by mixing 15 mL of a 0.015 % solution of lyophilised *E. coli* (strain W (ATCC) 9637; Sigma Ec9637), 15 mL of a 1 % yeast solution, and 45 μL of a 5 mg mL^{-1} solution of amphotericin B (Sigma A-9528) in distilled water, and either used immediately or stored overnight at 4°C.

A solution of test compound, or water as a negative control (2 μL), was added to each well, followed by agar (100 μL). The agar was allowed to set at room temperature, and then a solution of nematode eggs (60 μL; 100 eggs per 100 μL) and LDA media (40 μL) were added to each well. The plates were incubated for up to 10 days at 25°C in a plastic container, with a lid covering ~70 % of the opening. The larvae were aerated by blowing air over the plate following 24 h, and thereafter on every third day until the plates were evaluated.

Compounds **1** and **39** were also screened for anti-parasitic activity against the helminth *Heligmosomoides polygyrus*, in mice. Mice were infected with 100 *Heligmosomoides polygyrus* L3 larvae by oral gavage. Approximately 10 days later, infection was confirmed by faecal egg count. The infected mice were dosed with either compound **1** or **39** at 100 μM, or a DMSO control, by oral gavage based on bodyweight. After 7 days, the mice were killed and their intestines removed. The contents of the small intestines were flushed out with 5 mL of water into a Petri-dish (using a syringe). Adult worms were identified and counted using a dissecting microscope.

Supplementary Material

Experimental methods and compound characterization are available on the Journal's website.

Acknowledgements

The authors thank ParaCo Technologies Limited, New Zealand (whose interests in this study are protected under US patent application 61/468, 317) for their financial support.

References

[1] D. M. Leathwick, R. B. Besier, *Vet. Parasitol.* **2014**, *204*, 44. doi:10.1016/J.VETPAR.2013.12.022

[2] P. J. Waller, *Vet. Parasitol.* **2006**, *139*, 1. doi:10.1016/J.VETPAR.2006.02.036

[3] N. C. Sangster, *Int. J. Parasitol.* **1999**, *29*, 115. doi:10.1016/S0020-7519(98)00188-X

[4] Y. Bansal, O. Silikari, *Bioorg. Med. Chem.* **2012**, *20*, 6208. doi:10.1016/J.BMC.2012.09.013

[5] M. L. Fascio, M. I. Errea, N. B. D'Accorso, *Eur. J. Med. Chem.* **2015**, *90*, 666. doi:10.1016/J.EJMECH.2014.12.012

[6] A. Awasthi, M. Razzak, R. Al-Kassas, J. Harvey, S. Garg, *Chem. Pharm. Bull.* **2012**, *60*, 931. doi:10.1248/CPB.C12-00258

[7] E. Papadopoulos, E. Gallidis, S. Ptochos, *Vet. Parasitol.* **2012**, *189*, 85. doi:10.1016/J.VETPAR.2012.03.036

[8] I. A. Sutherland, D. M. Leathwick, *Trends Parasitol.* **2011**, *27*, 176. doi:10.1016/J.PT.2010.11.008

[9] A. Harder, G. von Samson-Himmelstjerna, *Parasitol. Res.* **2002**, *88*, 481. doi:10.1007/S00436-002-0619-2

[10] R. Kaminsky, P. Ducray, M. Jung, R. Clover, L. Rufener, J. Bouvier, S. S. Weber, A. Wenger, S. Wieland-Berghausen, T. Goebel, N. Gauvry, F. Pautrat, T. Skripsky, O. Froelich, C. Komoin-Oka, B. Westlund, A. Sluder, P. Maser, *Nature* **2008**, *452*, 176. doi:10.1038/NATURE06722

[11] R. B. Besier, *Trends Parasitol.* **2007**, *23*, 21. doi:10.1016/J.PT.2006.11.004

[12] T. G. Geary, N. C. Sangster, D. P. Thompson, *Vet. Parasitol.* **1999**, *84*, 275. doi:10.1016/S0304-4017(99)00042-4

[13] A. Rieche, H. Gross, E. Höft, *Org. Synth.* **1967**, *47*, 1. doi:10.15227/ORGSYN.047.0001

[14] A. Vilsmeier, A. Haack, *Ber. Dtsch. Chem. Ges.* **1927**, *60*, 119.

[15] A. A. Haddach, A. Kellemanb, M. V. Deaton-Rewolinskia, *Tetrahedron Lett.* **2002**, *43*, 399. doi:10.1016/S0040-4039(01)02192-X

[16] H.-J. Knölker, K. R. Reddy, *Chem. Rev.* **2002**, *102*, 4303. doi:10.1021/CR020059J

[17] P. Molina, P. M. Fresneda, P. Almendros, *Tetrahedron* **1993**, *49*, 1223. doi:10.1016/S0040-4020(01)85813-0

[18] A. W. Schmidt, K. R. Reddy, H.-J. Knölker, *Chem. Rev.* **2012**, *112*, 3193. doi:10.1021/CR200447S

[19] Y. M. Kim, S. Yu, *J. Am. Chem. Soc.* **2003**, *125*, 1696. doi:10.1021/JA028966T

[20] A. W. Freeman, M. Urvoy, M. E. Criswell, *J. Org. Chem.* **2005**, *70*, 5014. doi:10.1021/JO0503299

[21] T. Iihama, J.-M. Fu, V. Snieckus, *Synthesis* **1989**, 184. doi:10.1055/S-1989-27189

[22] M. Brandl, M. S. Weiss, A. Jabs, J. Suhnel, R. J. Hilgenfeld, *Mol. Biol.* **2001**, *307*, 357. doi:10.1006/JMBI.2000.4473

[23] E. A. Meyer, R. K. Castellano, F. Diederich, *Angew. Chem. Int. Ed.* **2003**, *42*, 1210. doi:10.1002/ANIE.200390319

[24] J. P. Gallivan, D. A. Dougherty, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 9459. doi:10.1073/PNAS.96.17.9459

[25] W. W. Limburg, J. F. Yanus, D. J. Williams, A. O. Goedde, J. M. Pearson, *J. Polym. Sci. A1* **1975**, *13*, 1133.

[26] M. Pudlo, D. Csanyi, F. Moreau, G. Hajos, Z. Riedl, J. Sapi, *Tetrahedron* **2007**, *63*, 10320. doi:10.1016/J.TET.2007.07.068

[27] G. C. Coles, C. Bauer, F. H. M. Borgsteede, S. Geerts, T. R. Klei, M. A. Taylor, P. Waller, *Vet. Parasitol.* **1992**, *44*, 35. doi:10.1016/0304-4017(92)90141-U