DOCTOR OF PHILOSOPHY

Haemanthamine, zephycandidine and quorum sensing N-Acyl homoserine lactones

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Haemanthamine, zephycandidine and quorum sensing *N*-Acyl homoserine lactones

A Thesis of requirements for the

Doctor of Philosophy

In the School of Natural Sciences

by

Jamie Tibble

Prifysgol Bangor • Bangor University

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I would like to thank my supervisor Dr Patrick Murphy for his experience and wisdom, the Murphy project group for their companionship and help, the technical and academic staff for their aid and guidance and lastly my fiancée Hayley for her continued support. Finally, Joel.

Abbreviations

Ac	Acetate
AHL	Acyl-Homoserine Lactone
AUR	Auranofin
BCVFA	Branched-Chain Volatile Fatty Acids
Bu	Butyl
d	Doublet
DCM	Dichloromethane
DDP	DDP-Cisplatin
DMAP	*N*, *N*-Dimethyl aminopyridine
DNA	Deoxyribonucleic Acid
Flu	Fluorene
m	Multiplet
Me	Methyl
Nic	Nicotinic Acid
Pr	Propyl
PQZ	Praziquantel
q	Quartet
QS	Quorum Sensing
Rac	Racemic
s	Singlet
t	Triplet

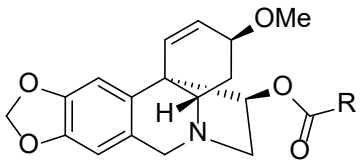
THF	Tetrahydrofuran
VFA	Volatile Fatty Acids
WHO	World Health Organisation

Abstract

This thesis is separated into three sections:

1) *Slight changes in the chemical structure of haemanthamine greatly influence the effect of the derivatives on rumen fermentation in vitro*

Nine compounds (**i**-**ix**) were synthetized from the natural alkaloid haemanthamine and tested in vitro for their effects on rumen protozoa and fermentation parameters. Our results showed that acetate, butyrate, pivalate or hexanoate derivatives of haemanthamine or dihydrohaemanthamine (**v**) gave compounds that had significant differences in their effect on rumen fermentation.



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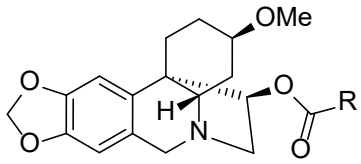
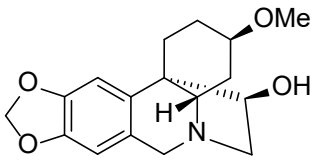
ii R= *t*-Bu
iii R= *n*-Pr
iv R= *n*-C_5H_{11}



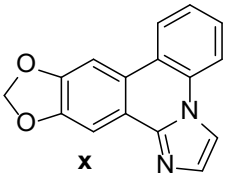
vi R= Me
vii R= *t*-Bu
viii R= *n*-Pr
ix R= *n*-C_5H_{11}



v

2) *Synthesis of zephycandidine A from haemanthamine*

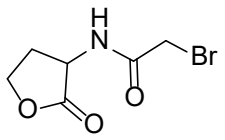
Zephycandidine A (**x**) the first naturally occurring imidazo[1,2–f]phenanthridine alkaloid, isolated from *Zephyranthes candida* (*Amaryllidaceae*) was prepared in three steps from the naturally occurring alkaloid haemanthamine.



x

3) *Quorum sensing* N-*Acyl homoserine lactones are a new class of anti-schistosomal agents*

Through evaluating and screening fourteen compounds (initially developed for anti-cancer and anti-viral projects) against *Schistosoma mansoni*, one of three species responsible for most cases of human schistosomiasis, a racemic N-acyl homoserine derivative demonstrated good efficacy against all intra mammalian lifecycle stages including schistosomula (EC50 = 4.7 µM), juvenile worms (EC50 = 4.3 µM) and adult worms (EC50 = 8.3 µM). To explore structural activity relationships, a further 8 analogues of this initial compound (**xi**-**xx**) were generated, including the separate (R)- (**xv**) and (S)-enantiomers (**xvi**). Anti-schistosomal screening of these analogues demonstrated that the (R)-enantiomer retained activity, whereas the (S)- was far less activity. Furthermore, modification of the lactone ring to a thiolactone ring improved potency against schistosomula (EC50 = 2.1 µM), juvenile worms (EC50 = 0.5 µM) and adult worms (EC50 = 4.8 µM). As the effective racemic parent compound is structurally similar to quorum sensing signalling peptides used by bacteria, further evaluation of its effect (along with its stereoisomers and the thiolactone analogues) against Gram+ (*Staphylococcus aureus*) and Gram- (*Escherichia coli*) species was conducted. While some activity was observed against both Gram+ and Gram- bacteria species for the racemic compound (MIC 125 mg/L), the (R)-stereoisomer had better activity (125 mg/L) than the (S) (>125mg/L). However, the greatest antimicrobial activity (MIC 31.25 mg/L against *S. aureus*) was observed for the thiolactone containing analogue.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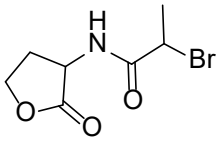
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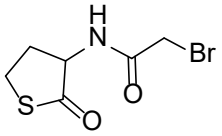
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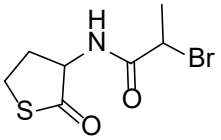
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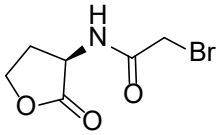
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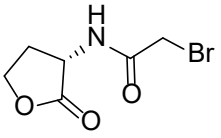
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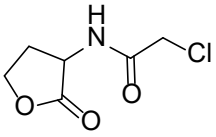
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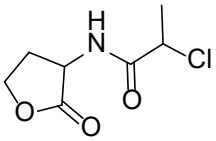
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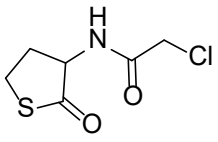
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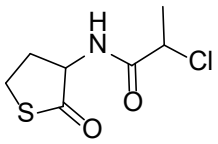
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Chapter 1

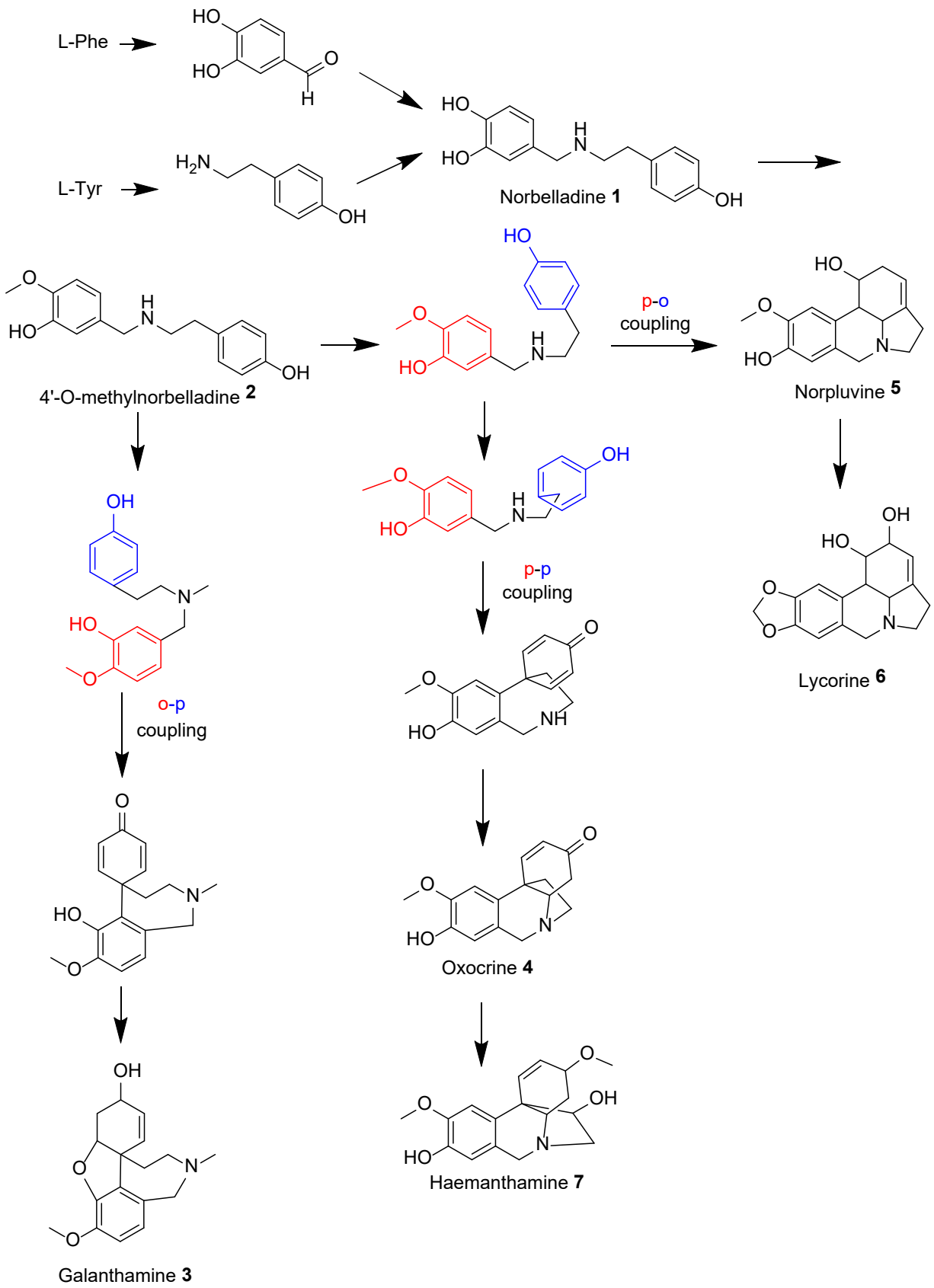
"Slight changes in the chemical structure of haemanthamine greatly influence the effect of the derivatives on rumen fermentation *in vitro*"

Eva Ramos-Morales, Jamie Tibble-Howlings, Laura Lyons, Magnus O. Ogbu, Patrick J. Murphy, Radek Braganca and Charles James Newbold.

Sci Rep **9,** 2440 (2019). https://doi.org/10.1038/s41598-019-38977-x

Introduction

The daffodil family contains a series of alkaloids known as the *amaryllidaceae* alkaloids (*amaryllidaceae* being the family of flowering plants, perennials that grow from bulbs), including norbelladine **1**, galanthamine **3**, norpluvine **5,** lycorine **6**, haemanthamine **7**. Each of these alkaloids has a similar biosynthetic pathway, coming from L-phenylalanine and L-tyrosine (Scheme 1). Galanthamine **3** coming from an *ortho-para* coupling, haemanthamine **7** coming from a *para-para* free radical coupling and lycorine **6** from a *para-ortho* free radical coupling (Scheme 1).



Scheme 1: The biosynthetic pathway of some of the *amaryllidaceae* alkaloids.

Over the years the Murphy group[1,2] has investigated the isolation and derivatisation of haemanthamine **7** (Figure 1), and its synthetic product of hydrogenation, dihydrohaemanthamine **8** (Figure 2).

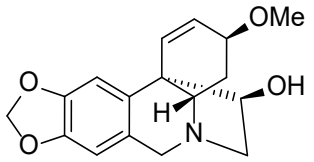


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Figure 1: Haemanthamine **7.**

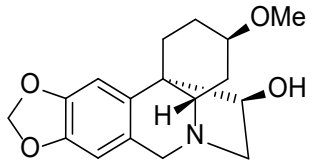

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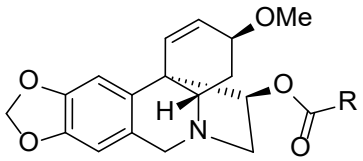
Figure 2: Dihydrohaemanthamine **8.**

A number of compounds have already been synthesised; these include compounds **9**-**16**[2] (Figure 3).



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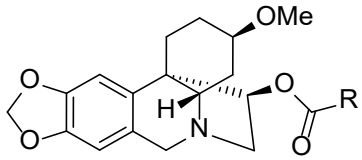
10 R= *t*-Bu
11 R= *n*-Pr
12 R= *n*-C_5H_{11}



13 R= Me
14 R= *t*-Bu
15 R= *n*-Pr
16 R= *n*-C_5H_{11}

Figure 3: Compounds **9**-**16.**

Haemanthamine can be extracted from the crude residues of an industrial galantamine extraction procedure (Figure 4), implemented by Agroceutical Products LTD. Previous work from Ogbu[3], Bernitzky[4], Seabrook[1] and Feil[5] gave methodology for extraction of haemanthamine (ca. 8-12% of total alkaloids remaining) from this residue, including some methods that were abandoned, such as using supercritical CO_2,[5] which yielded poor results. During the research of Ogbu a more effective extraction method was discovered and implemented for extraction of haemanthamine from the crude source, and this method is outlined in the experimental section of this report. Experimentation into this method concluded that some of these steps were unnecessary and the process could be simplified, as well as certain material components being unnecessary for the extraction. NMR spectroscopy

and TLC analysis determined that the haemanthamine extracted in the modified method yielded haemanthamine of higher purity, with fewer consumables and, therefore, an overall cheaper and easier process.

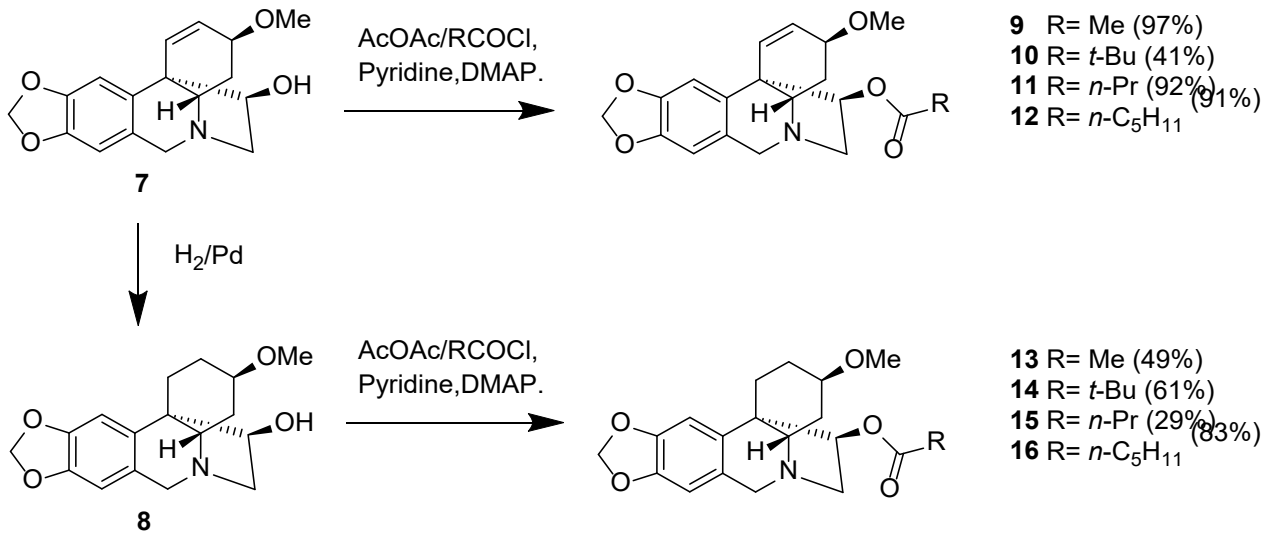
Figure 4: Crude runoff from galantamine extraction.

The extraction method used within the group used many unrecyclable materials such as Celite®, silica and filter papers, the aim of this project was to minimise these unnecessary expenditures while maximising the haemanthamine isolation. Experimentation into the extraction gave rise to the conclusion that the use of Celite® was not necessary and was only adding to the cost of the extraction, the same result could be accomplished by a sheet of filter paper via vacuum filtration or a plug of cotton wool using gravity filtration. These first insights were the driving force for the continuation of this project, making minor modificiations to find the best method possible. It was also discovered that the volumes of solvents in this extraction were excessive, from the volume of chloroform used to the volumes of recrystallization liquors. Thus, reducing the excessive use of these gave a more manageable system and recycling of these solvents gave way to an easy and fast extraction procedure that costs very little to undertake. The quantity of base used in the experiments was also altered but found to be only slightly in excess, this being one of the only materials not recyclable in the process.

Compounds **9-16** were synthesised for a previous project[2] and tested as to their effectiveness against protozoan organisms, and for their potential to reduce methane production in the rumen of farmed cattle[6]. This work was performed in collaboration with the research group of Professor J. Newbold at Aberystwyth University.

It is estimated that dairy cattle in the UK produce around 230,000 tons of methane gas from flatulence each year[7]. Cows belong to the class of mammal called ruminants; these mammals all store their ingested food in the first chamber of their stomach, called the rumen. The rumen in these animals contains many bacterial microorganisms, some of which produce methane as a by-product[6]. Methane escapes from the cattle in the form of belching and/or flatulence, travelling into the atmosphere and becoming trapped there. Methane gas is known as a 'greenhouse gas', meaning that the release of methane into the atmosphere leads to the trapping of heat from the sun's radiation, thus causing global temperatures to rise gradually over time. Methane traps warming radiation within the atmosphere around thirty times more effectively than carbon dioxide[8], which is the dominant greenhouse gas produced by man. Although methane persists in the atmosphere for a shorter period of time than carbon dioxide[9], its warming effect in the short term could cause immense damage to the environment, changing global climate at a rapid rate. Current attempts at reversing the effects of global warming may well be in vain if the production of greenhouse gasses continue at their current pace. Reducing the production of methane in the atmosphere will, however, not halt global warming, reduction of CO_2 would be the most appropriate way to attempt to fight back against climate change. However, reduction of methane gas is a good start, easing the strain on the environment a small amount to allow time for new energy sources to be produced that have low carbon emissions.

Results and Discussion



Scheme 2: Synthesis of **8**-**16** from haemanthamine **7**.

The two main synthetic methods used in these projects were ester synthesis from secondary alcohols and either and acyl chloride or acid anhydride, and hydrogenation reaction using catalytic palladium on carbon and hydrogen gas.

Compounds **7** and **8**-**16** were sent to Dr Newbold at Aberystwyth University for an array of testing in an artificial cow rumen: pH tests, volatile fatty acid concentration (VFA), acetate percentage, propionate percentage, butyrate percentage, branched-chain volatile fatty acid percentage (BCVFA), ammonia production, total gas production and methane production.

The testing at Aberystwyth was carried by the Newbold group, using an artificial cow rumen (a procedure in which I assisted in the initial stages) produced by taking cow rumen contents, mixing them with sufficient feed and general supplements that the animals would receive in the farm. This mixture was then allowed to propagate and then separated into a series of artificial rumens, each a sealed system. The individual systems were then treated with varying concentrations of alkaloid derivatives and allowed to ferment over a period of time. The contents were then tested vs a control for the various conditions listed above.

Effects of compounds 7-16 on the pH of the artificial rumen system.

The average pH of the bovine rumen ranges from between 5.7 and 7.3 and, as can be seen from Table 2, the pH of the artificial rumen changes within normal pH parameters with the addition of any of the haemanthamine derivatives (**7**, **9-16**). The changes are still within the ranges of the average rumens, so therefore should not negatively impact any live cattle in this respect.

	pH				
	0 g/L	**0.5 g/L**	**1 g/L**	**SED**	**P**
7	6.20	6.25	6.36	0.028	0.003
9	6.20	6.23	6.37	0.031	0.004
10	6.20	6.17	6.37	0.026	<0.001
11	6.20	6.30	6.38	0.031	0.003
12	6.20	6.29	6.27	0.032	0.062
13	6.20	6.32	6.34	0.038	0.017
14	6.20	6.31	6.33	0.023	0.002
15	6.20	6.33	6.37	0.034	0.005
16	6.20	6.26	6.26	0.046	0.339

Table 1: The effect of compound **7-16** on the pH of the system.

SED: Standard error of the difference. P: P-Value

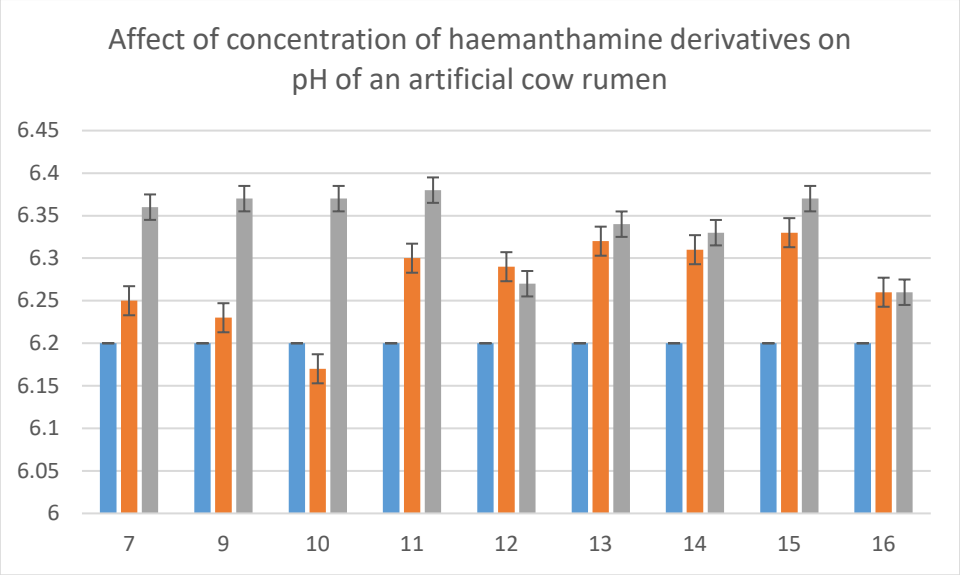


Table 2: Effect of compounds **7-16**, added at 0.0 (Blue: control) 0.5 (Orange) or 1.0 (Grey) g/L, on pH after 24 h of incubation (batch culture).

The SED and P values both show that these results are statistically significant. As mentioned, the pH range of the average cow rumen is between 5.7 and 7.3 meaning that the maximum change of 0.18 is fairly insignificant, with respect to the overall system.

Effect of compounds 7-16 on production of VFAs in the artificial rumen system

Volatile fatty acids (VFA) are used to provide energy to the rumen and are used in milk fat biosynthesis. They are the main energy source for ruminants, providing approx. 70% of their total energy requirements. The three main VFAs found in the rumen are acetates, propionates and butyrates.

	VFA Production (mM)				
	0 g/L	**0.5 g/L**	**1 g/L**	**SED**	**P**
7	62.9	57.7 (92%)	52.6 (84%)	2.28	0.012
9	62.9	50.7 (81%)	46.7 (74%)	2.38	0.01
10	62.9	55.6 (88%)	52.5 (83%)	2.18	0.008
11	62.9	52.1 (83%)	49.0 (78%)	1.97	<0.001
12	62.9	51.7 (82%)	53.3 (85%)	1.56	<0.001
13	62.9	51.1 (81%)	49.5 (79%)	1.89	<0.001
14	62.9	51.3 (82%)	55.6 (88%)	3.29	0.033
15	62.9	51.4 (82%)	46.8 (74%)	4.98	0.044
16	62.9	55.6 (88%)	62.3 (99%)	3.25	0.117

Table 3: The effect of compounds **7-16** on VFA production in the system vs control (%). SED: Standard error of difference.

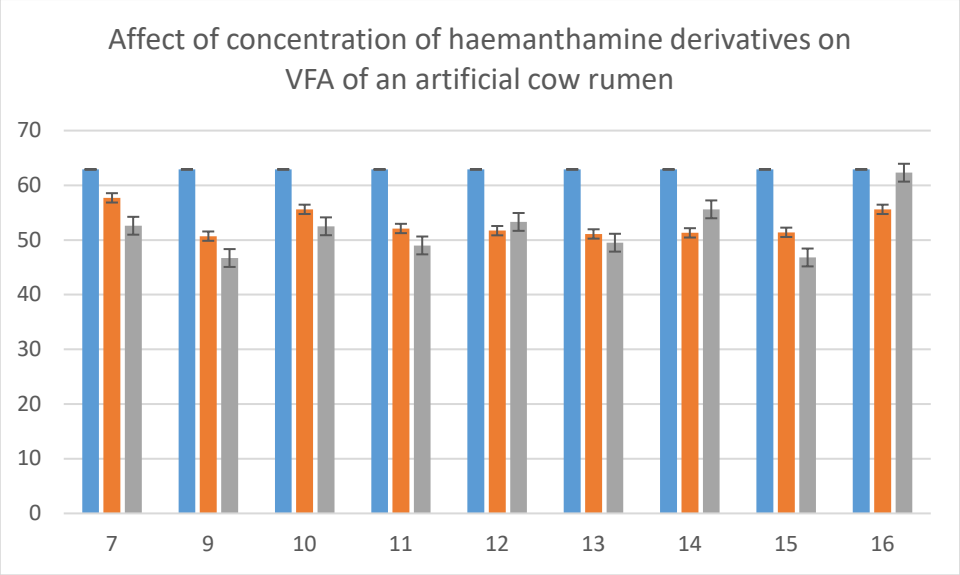


Table 4: Effect of **7-16,** added at 0.0 (Blue: control) 0.5 (Orange) or 1.0 (Grey) g/L, on total VFA (mM) after 24 h of incubation (batch culture).

The addition of each compound has a negative effect on the total amount of VFAs produced in the rumen, between a 1-26% reduction vs the control. This could have a significantly negative effect on the ruminant as they rely on the VFAs for their main source of energy.

Effect of compounds 7-16 on acetate production in the artificial rumen system

Acetates are one of the VFAs in the bovine rumen, the haemanthamine derivatives do seem to have a negative effect on the production of acetates in the artificial rumens. This may be problematic for live bovines; however further testing is needed.

	Acetate Production (% of total VFA)				
	0 g/L	**0.5 g/L**	**1 g/L**	**SED**	**P**
7	58.8	56.4 (94%)	55.5 (94%)	1.94	0.287
9	58.8	48.0 (82%)	47.5 (81%)	1.36	<0.001
10	58.8	48.7 (83%)	46.0 (78%)	3.131	0.015
11	58.8	47.3 (80%)	42.9 (73%)	0.925	<0.001
12	58.8	48.3 (82%)	46.6 (79%)	0.837	<0.001
13	58.8	49.0 (83%)	47.5 (81%)	0.936	<0.001
14	58.8	44.6 (76%)	41.6 (71%)	0.804	<0.001
15	58.8	45.5 (77%)	36.8 (63%)	0.833	<0.001
16	58.8	41.3 (70%)	41.2 (70%)	1.399	<0.001

Table 5: The effect of compounds **7**-**16** on total acetate production in the system vs control (%). SED: Standard error of difference.

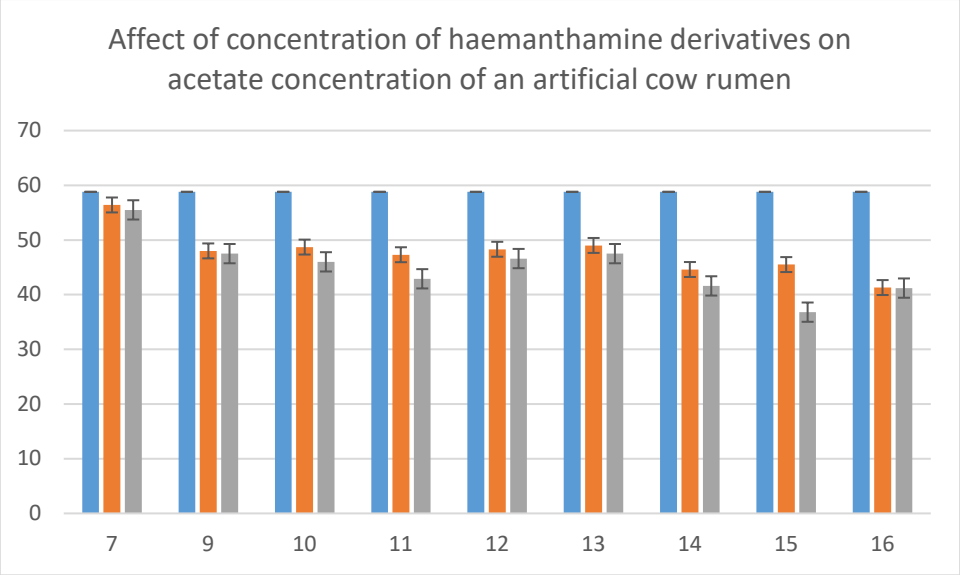


Table 6: Effect of compounds **7-16**, added at 0.0 (Blue: control) 0.5 (Orange) or 1.0 (Grey) g/L, on acetate (%) after 24 h of incubation (batch culture).

This graph shows that additions of these ester compounds have a negative effect on the total concentration of acetate VFA's in the rumen, leading to a reduction of between 6-37% of acetate production in the rumen. It is unknown as to what effect of this reduction will actually have on ruminants.

Effect of compounds 7-16 on propionate production in the artificial rumen system

Propionate concentration seems to be affected positively with the addition of the haemanthamine **7**, and derivatives, **9**-**16**, increasing the concentrations of propionates in a fairly consistent manner for all of the derivatives.

	Propionate Production (% of total VFA)				
	0 g/L	**0.5 g/L**	**1 g/L**	**SED**	**P**
7	16.4	19.8 (121%)	20.2 (123%)	1.22	0.039
9	16.4	24.6 (150%)	23.8 (145%)	0.749	<0.001
10	16.4	23.2 (141%)	23.9 (146%)	1.27	0.002
11	16.4	23.3 (142%)	25.0 (152%)	0.645	<0.001
12	16.4	23.3 (142%)	23.6 (144%)	0.426	<0.001
13	16.4	25.0 (152%)	24.5 (149%)	0.457	<0.001
14	16.4	26.2 (160%)	33.9 (207%)	0.788	<0.001
15	16.4	24.0 (146%)	26.8 (163%)	0.392	<0.001
16	16.4	27.2 (166%)	37.9 (231%)	0.808	<0.001

Table 7: The effect of compounds **7**-**16** on total propionate production in the system vs control (%). SED: Standard error of difference.

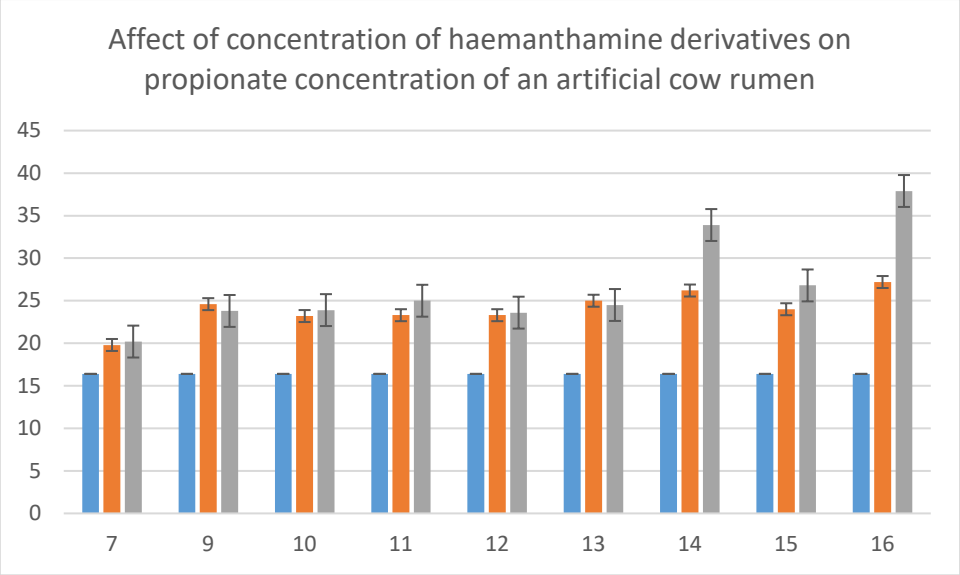


Table 8: Effect of compounds **7-16**, added at 0.0 (Blue: control) 0.5 (Orange) or 1.0 (Grey) g/L, on propionate (%) after 24 h of incubation (batch culture).

Thus, in contrast to the previous study, the production of propionate VFAs shows a net increase after the addition of the compounds, ranging from a 21-107% increase.

Effect of compounds 7-16 on butyrate production in the artificial rumen system

Butyrates are variably affected by the addition of the haemanthamine derivatives with some of the compounds resulting in an increased production, and others leading to a reduction.

	Butyrate Production (% of total VFA)				
	0 g/L	**0.5 g/L**	**1 g/L**	**SED**	**P**
7	18.5	17.8 (96%)	18.3 (99%)	0.747	0.689
9	18.5	21.3 (115%)	22.3 (121%)	0.647	0.003
10	18.5	21.9 (118%)	23.6 (128%)	1.864	0.081
11	18.5	22.8 (124%)	25.8 (139%)	0.418	<0.001
12	18.5	21.2 (115%)	22.2 (120%)	0.585	0.002
13	18.5	19.6 (106%)	21.8 (118%)	0.608	0.004
14	18.5	22.0 (119%)	17.0 (92%)	1.198	0.015
15	18.5	24.0 (130%)	29.2 (158%)	0.77	<0.001
16	18.5	22.3 (121%)	10.5 (57%)	1.322	<0.001

Table 9: The effect of compounds **7**-**16** on total butyrate production in the system vs control (%). SED: Standard error of difference.

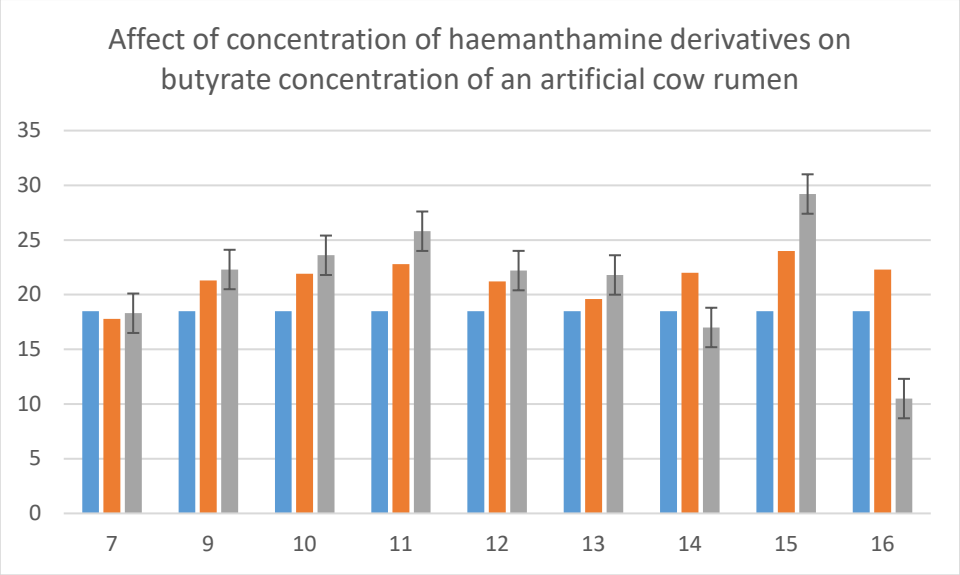


Table 10*: Effect of compounds* **7-16***, added at 0.0 (Blue: control) 0.5 (Orange) or 1.0 (Grey) g/L, on butyrate (%) after 24 h of incubation (batch culture).*

The concentration of butyrates seems to increase with the addition of each compound, giving a maximum increase of 58%, except **14** and **16** which had a maximum reduction of butyrates by 43%. Overall, the majority of the changes are minimal with the exception of compound **16**.

Effect of compounds 7-16 on BCVFA production in the artificial rumen system

Branched-chain volatile fatty acids (BCVFA) are produced in the rumen of the cattle for the cellulolytic microorganisms that inhabit the organ. These BCVFAs are produced from dietary proteins or from the recycling of bacterial proteins. The most common BCVFAs are isobutyric acid, isovaleric acid, valeric acid and 2-methylbutyric acid.

	BCVFA Production (% of total VFA)				
	0 g/L	**0.5 g/L**	**1 g/L**	**SED**	**P**
7	2.70	2.47 (91%)	2.49 (92%)	0.076	0.036
9	2.70	2.56 (95%)	2.56 (95%)	0.103	0.352
10	2.70	2.49 (92%)	2.41 (89%)	0.042	0.001
11	2.70	2.68 (99%)	2.55 (94%)	0.06	0.079
12	2.70	2.79 (103%)	2.52 (93%)	0.088	0.054
13	2.70	2.64 (98%)	2.45 (91%)	0.099	0.097
14	2.70	3.27 (121%)	4.18(178%)	0.185	<0.001
15	2.70	2.58 (96%)	2.05 (76%)	0.176	0.021
16	2.70	2.28 (84%)	2.05 (76%)	0.124	0.005

Table 11: The Effect of compounds **7**-**16** on total BCVFA production in the system vs control (%). SED: Standard error of difference.

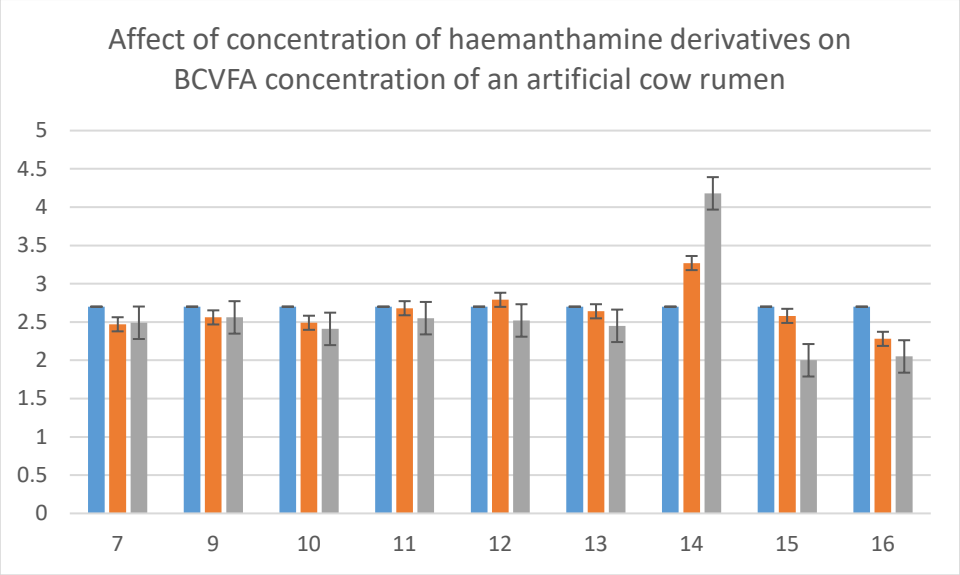


Table 121: Effect of compounds **7-16**, added at 0.0 (Blue: control) 0.5 (Orange) or 1.0 (Grey) g/L, on BCVFA (%) after 24 h of incubation (batch culture).

Each compound in this test, apart from **14**, led to a reduction of the BCVFAs in the system, the biggest change being a 24% reduction in compound **16**. According to the SED and P values each of these results appears to be statistically significant.

Effect of compounds 7-16 on ammonia production in the artificial rumen system

Ammonia production in the rumen seems to reduce with the addition of the haemanthamine derivatives, with redutctions ranging from 1-28%. Compound **9** gave the greatest reduction in ammonia production, 28%; whereas compound **12** gave the lowest.

	Ammonia Production (mM)				
	0 g/L	**0.5 g/L**	**1 g/L**	**SED**	**P**
7	7.12	5.38 (76%)	5.85 (82%)	0.195	<0.001
9	7.12	5.16 (72%)	6.06 (85%)	0.431	0.011
10	7.12	5.24 (74%)	6.17 (87%)	0.349	0.005
11	7.12	6.51 (91%)	6.34 (89%)	0.167	0.008
12	7.12	7.04 (99%)	6.06 (93%)	0.313	0.027
13	7.12	5.72 (80%)	6.12 (86%)	0.297	0.008
14	7.12	6.94 (97%)	6.72 (94%)	0.454	0.686
15	7.12	6.65 (93%)	6.76 (95%)	0.234	0.184
16	7.12	6.86 (96%)	6.08 (85%)	0.278	0.023

Table 13: The effect of compounds **7**-**16** on total ammonia production in the system vs control (%). SED: Standard error of difference.

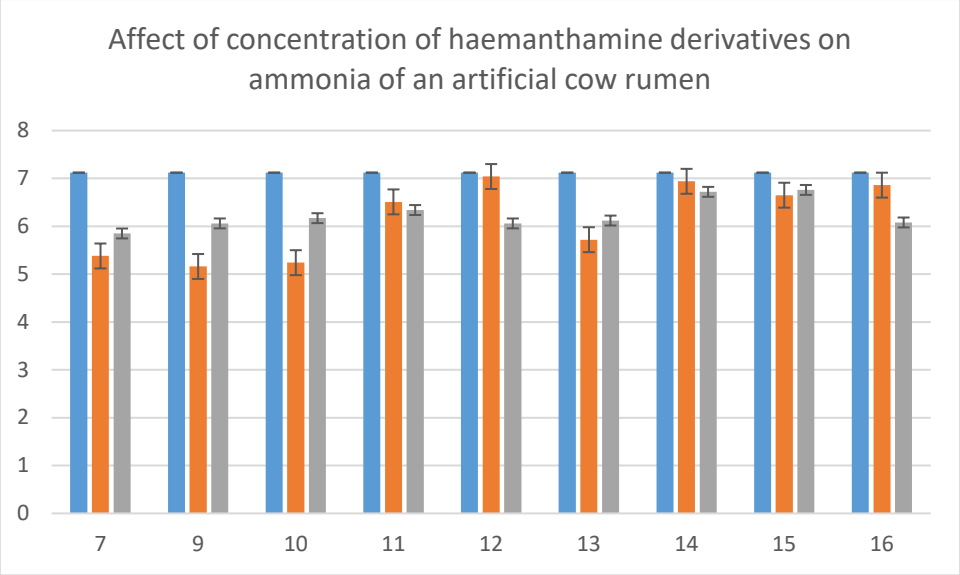


Table 14: Effect of compounds **7-16**, added at 0.0 (Blue: control) 0.5 (Orange) or 1 (Grey) g/L, on ammonia (mM) after 24 h of incubation (batch culture).

Effect of compounds 7-16 on total gas evolution from the artificial rumen system

Total gas evolution is decreased with the addition of haemanthamine derivatives, this could be from the stop in production of methane gas as described in Table 15.

	Total gas evolution (mL)				
	0 g/L	**0.5 g/L**	**1 g/L**	**SED**	**P**
7	25.1	22.0 (88%)	19.0 (76%)	0.917	0.002
9	25.1	21.4 (85%)	18.0 (72%)	1.331	0.006
10	25.1	25.6 (102%)	17.8 (71%)	1.327	0.003
11	25.1	22.1 (88%)	19.8 (79%)	0.943	0.004
12	25.1	22.1 (88%)	21.6 (86%)	1.371	0.089
13	25.1	21.8 (87%)	19.6 (78%)	0.842	0.004
14	25.1	20.6 (82%)	17.8 (71%)	0.742	<0.001
15	25.1	21.0 (84%)	19.8 (79%)	0.947	0.003
16	25.1	19.4 (77%)	18.4 (73%)	2.036	0.034

Table 15: The effect of compounds **7**-**16** on total gas evolved by the system vs control (%).

SED: Standard error of difference.

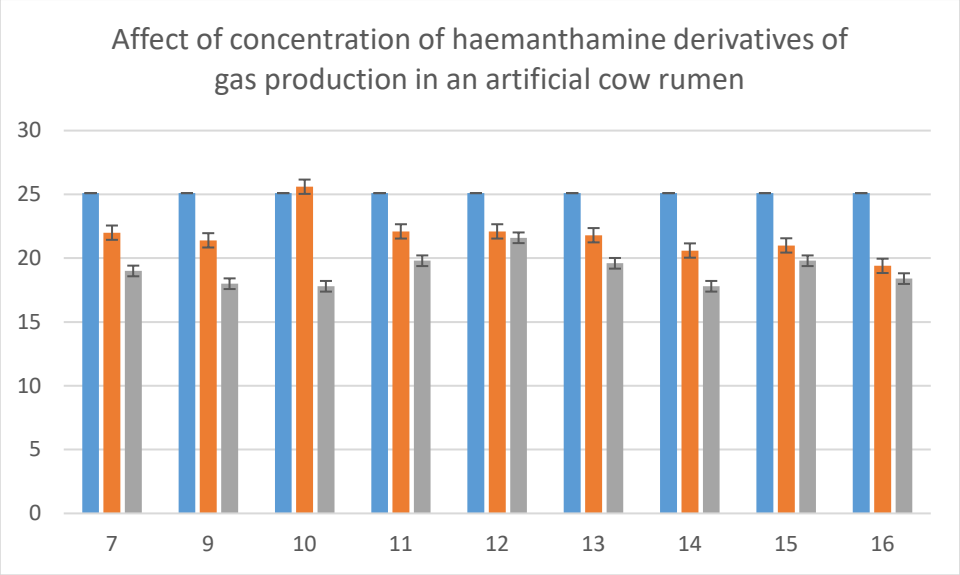


Table 16: Effect of compounds **7-16**, added at 0.0 (Blue: control) 0.5 (Orange) or 1.0 (Grey) g/L, on total gas (mL) produced after 24 h of incubation (batch culture).

Effect of compounds 7-16 on methane production in the artificial rumen system

The drop in methane production with the addition of derivatives is astonishingly high. The reduction seen with **7** is the least effective of the derivatives, lowering methane production by around 35-40%, followed by **10,** which lowers the production by around 84-90%, while compounds **9**, **11**, **12**, **13**, **14**, **15** and **16** lower the production by over 99%.

	Methane evolution (mL)				
	0 g/L	**0.5 g/L**	**1 g/L**	**SED**	**P**
7	1.19	0.774(65%)	0.650 (55%)	0.300	0.242
9	1.19	0.002 (0%)	0.001 (0%)	0.100	<0.001
10	1.19	0.399 (28%)	0.193 (16%)	0.206	0.0066
11	1.19	0.001 (0%)	0.001 (0%)	0.100	<0.001
12	1.19	0.001 (0%)	0.001 (0%)	0.100	<0.001
13	1.19	0.001 (0%)	0.001 (0%)	0.100	<0.001
14	1.19	0.001 (0%)	0.001 (0%)	0.100	<0.001
15	1.19	0.001 (0%)	0.001 (0%)	0.100	<0.001
16	1.19	0.001 (0%)	0.001 (0%)	0.100	<0.001

Table 17: The effect of compounds **7**-**16** on total methane production in the system vs control (%). SED: Standard error of difference.

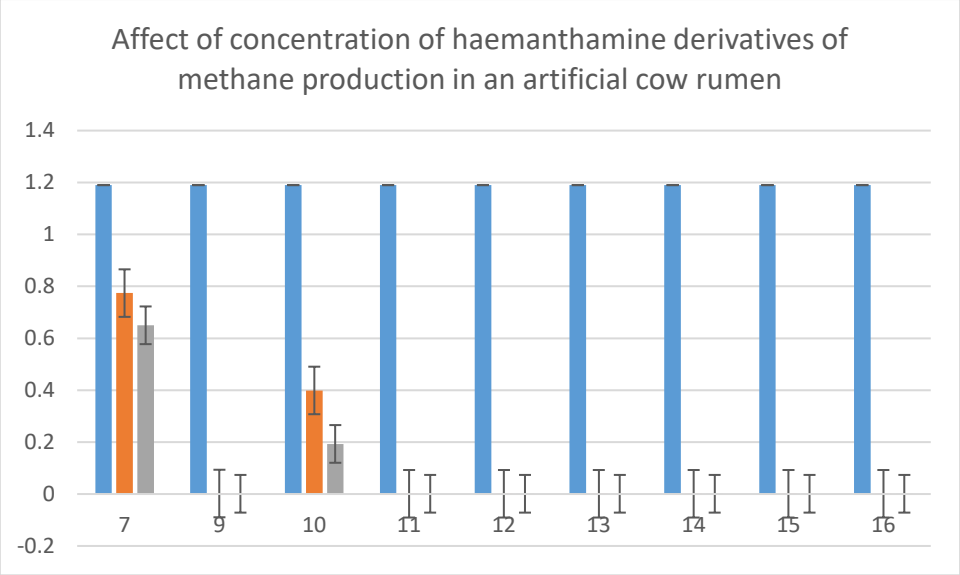


Table 18: Effect of compounds **7-16**, added at 0.0 (Blue: control) 0.5 (Orange) or 1.0 (Grey) g/L, on methane (mL) produced after 24 h of incubation (batch culture).

Overall conclusions

Haemanthamine and the derivatives caused a slight increase of the pH ($p < 0.05$) in comparison to the control. In addition, all treatments caused a reduction in the concentration of total VFA ($P < 0.05$) to different extent depending on the compound (between 8 and 25%).

Haemanthamine and the derivatives shifted the fermentation towards lower acetate ($P < 0.05$) and higher propionate ($P < 0.05$) production as compared with the control, with **12**, **13** and **14** having the strongest effect. However, butyrate concentration was not affected in the presence of haemanthamine ($P > 0.05$), most of the derivatives caused an increase ($P < 0.05$). Haemanthamine was more efficient in reducing the concentration of ammonia than most of the derivatives. A reduction in the total gas produced after 24 h was observed for all treatments. Finally, while haemanthamine decreased methane production by 35-45%, the other derivatives caused a reduction of 99.9%

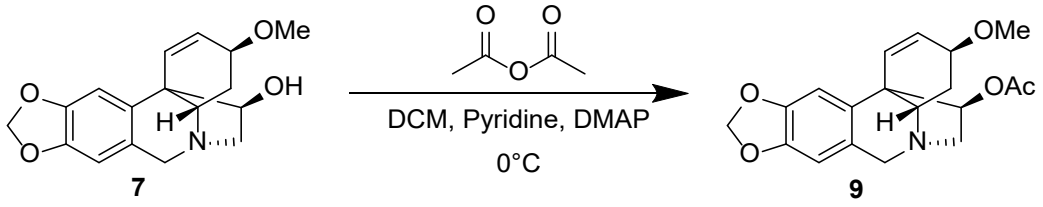
In conclusion, a strong effect on fermentation was observed in the presence of haemanthamine and its derivatives (decreases in total VFA and gas produced after 24 h). The derivatives seemed to cause the diversion of H_2 away from methane towards propionate and butyrate formation.

Experimental

General experimental synthesis and spectroscopy procedures

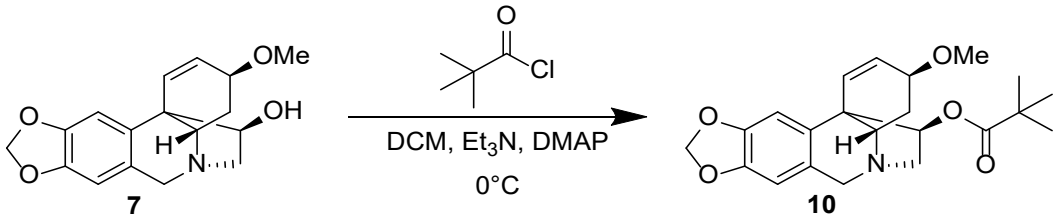
Reactions were stirred using a magnetic stirrer bar and monitored by TLC on Kieselgel 60 F254 silica gel coated glass plates, which were visualised by either I_2, UV light or phosphomolybdic acid. General chemicals were purchased from Sigma Aldrich, Alfa Aesar, Fluorochem or Fisher Scientific. Flash chromatography was carried out on Fluorochem Silica gel 60Å (40-63 micron) with an eluting solvent as specified. ^{1}H and ^{13}C NMR spectroscopic analyses were carried out on a Bruker Ultrashield Plus 400 MHz spectrometer in $CDCl_3$ unless otherwise stated and are reported in ppm referenced to the solvent internal standard at 7.26 and 77.160 ppm respectively. Infrared spectroscopic analyses were acquired on a Bruker Alpha ATR instrument. Mass spectra were recorded on either a Finnigan MAT 900 XLT or a Finnigan MAT 95 XP at the EPSRC National Mass Spectrometry Service Centre in Swansea.

(3*S*,4a*S*,5*S*,12*R*)-3-methoxy-4,4a-dihydro-3*H*,6*H*-11b,5-ethano[1,3]dioxolo[4,5-*j*]phenanthridin-12-yl acetate



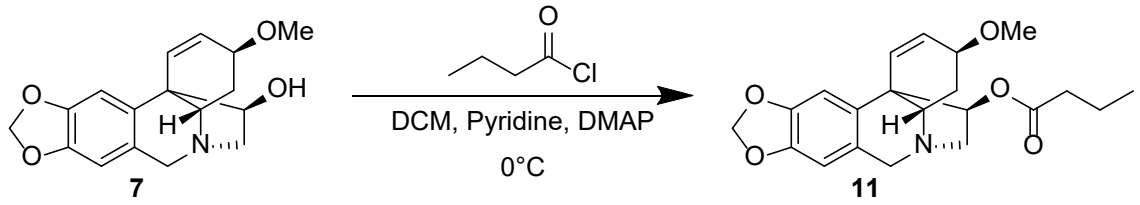
Pyridine (1.34 mL, 16.6 mmol) and DMAP (10 mg) were added to a solution of haemanthamine **7** (1.00 g, 3.32 mmol) dissolved in dichloromethane (20 mL). The mixture was cooled (0°C) and acetic anhydride (0.94 mL, 9.96 mmol) was added slowly. The resultant mixture was stirred until complete consumption of starting material (TLC; 5% MeOH in DCM, Rf = 0.54). The reaction was washed with aq. NaOH (1M, 3 x 30 mL) and brine (3 x 30 mL), dried ($MgSO_4$), filtered and evaporated under reduced pressure. The crude product was purified using silica gel flash chromatography (1-5 % MeOH in chloroform, gradient elution) to give **9** (1.10 g, 3.20 mmol, 97 % yield) as a gum. Rf = 0.48 (10% MeOH in DCM**)** $\boldsymbol{\delta_H}$ 6.89 (1H, s, CH), 6.46 (1H, s, CH), 6.34 (1H, d, *J* 10.1 Hz, CH), 6.16 (1H, dd, *J* 4.6, 10.1 Hz, CH), 5.89 (2H, s, CH_2), 4.96 (1H, dd, *J* 3.4, 7.0 Hz, CH), 4.35 (1H, d, *J* 16.8 Hz, CH), 3.83-3.86 (1H, m, CH), 3.73 (1H, d, *J* 16.8), 3.40 (1H, dd, *J* 7.2, 14.5, CH), 3.53 (3H, s, CH_3), 3.13 (1H, dd, *J* 3.6, 14.5 Hz, CH), 2.05 (1H, dd, *J* 4.5, 13.6, CH), 1.97 (3H, s, CH_3), 1.92 (1H, dd, *J* 5.2, 13.6 Hz, CH); $\boldsymbol{\delta_C}$ 170.2, 146.8, 146.6, 134.4, 129.7, 127.8, 126.5, 106.7, 104.1, 101.0, 80.5, 77.2, 72.6, 63.0, 61.3, 60.7, 56.7, 28.5, 21.4 ppm; $\boldsymbol{\nu_{max}}$ 2932, 2821, 1737, 1483, 1240, 1035, 754 cm^{-1}; **MS(CI)** 344.2 (100%, $[M+H^+]$) 687.3 (10%, $[2M+H^+]$); **HRMS(ES)** found 344.1490, $C_{19}H_{22}O_5N$ ($[M+H^+]$) requires 344.1492.

(3*S*,4a*S*,5*S*,12*R*)-3-methoxy-4,4a-dihydro-3*H*,6*H*-11b,5-ethano[1,3]dioxolo[4,5-*j*]phenanthridin-12-yl pivalate



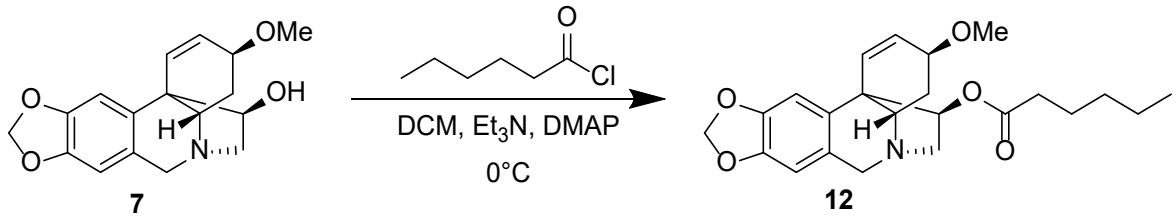
Triethylamine (1.39 mL, 9.96 mmol) and DMAP (10 mg) were added to a solution of haemanthamine **7** (1.0 g, 3.32 mmol) in dichloromethane (20 mL). The mixture was cooled (0°C), pivaloyl chloride (0.98 mL, 9.96 mmol) was added slowly and the mixture allowed to stir until complete consumption of the starting material (TLC; 10% MeOH in DCM Rf = 0.59). The sample was then washed with aq. NaOH (1M, 3 x 30 mL) and brine (3 x 30 mL) then dried (MgSO4), filtered and evaporated under reduced pressure. The product was then purified using flash column chromatography (1-10% MeOH in chloroform, gradient elution) to give **10** (520 mg, 1.35 mmol, 41 % yield) as a gum. Rf = 0.61 (10% MeOH in DCM); δ_H 6.91 (1H, s, CH), 6.47 (1H, s, CH), 6.35 (1H, d, *J* 10.1 Hz, CH), 6.15 (1H, dd, *J* 5.0, 10.0 Hz, CH), 5.89 (2H, s, CH2), 4.92 (1H,dd, *J* 3.3, 7.1 Hz, CH), 4.36 (1H, d, *J* 16.9 Hz, CH), 3.80-3.86 (1H, m, CH), 3.72 (1H, d, *J* 6.9 Hz, CH), 3.38-3.43 (1H, m, CH), 3.36 (3H, s, CH3), 3.26 (1H, dd, *J* 3.3, 14.3 Hz, CH), 2.05 (1H, dd, *J* 4.5, 13.7 Hz, CH), 1.94 (1H, td, *J* 4.2, 13.5 Hz, CH), 1.14 (9H, s, 3 x CH_3); δ_C 177.5, 146.7**,** 146.5, 134.4, 129.5, 127.9, 126.6, 106.7, 104.0, 101.0, 79.9, 72.8, 62.9, 61.3, 60.9, 56.6, 53.5, 49.3, 28.4, 27.2 ppm; ν_{max} 2930.19, 1724.68, 1483.32, 1155.56; **MS(CI)** 386.2 (100%, $[M+H^+]$) ; **HRMS(ES)** found 386.1954, $C_{22}H_{28}O_5N$ ($[M+H^+]$) requires 386.1962

(3*S*,4a*S*,5*S*,12*R*)-3-methoxy-4,4a-dihydro-3*H*,6*H*-11b,5-ethano[1,3]dioxolo[4,5-*j*]phenanthridin-12-yl butyrate



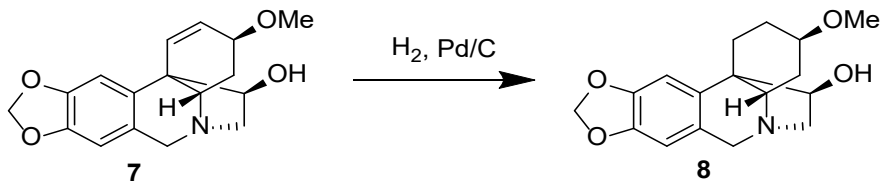
Using method from **4**, **7** (1.0 g, 3.32 mmol) was dissolved in dichloromethane (30 mL) and treated with pyridine (1.34 mL, 16.6 mmol), DMAP (10 mg) and butanoyl chloride (0.67 mL, 5.97 mmol). The product was then purified using flash column chromatography (2-5% gradient eluention of MeOH in chloroform to give **11** (1.125 g, 3.03 mmol, 92 % yield) as a gum. Rf = 0.53 (10% MeOH in DCM); δ_H 6.88 (1H, s, CH), 6.44 (1H, s, CH), 6.33 (1H, d, *J* 10.1 Hz, CH), 6.12 (1H, dd, *J* 5.0, 10.0 Hz, CH), 5.87 (2H, s, CH2), 4.96 (1H, dd, *J* 3.5, 7.1 Hz, CH), 4.33 (1H, d, *J* 16.9, CH), 3.83-3.79 (1H, m, CH), 3.69 (1H, d, *J* 16.9 Hz, CH), 3.41-3.53 (1H, m, CH), 3.34 (3H, s, CH3), 3.28 (1H, dd, *J* 3.5, 14.3 Hz, CH), 2.18 (2H, td, *J* 2.7, 7.4 Hz, CH2), 2.02 (1H, dd, *J* 4.8, 13.6 Hz, CH), 1.92 (1H, td, *J* 4.2, 13.4 Hz, CH), 1.57 (2H, hex, *J* 7.39 Hz, CH_3-**C**H_2-CH_2), 0.91 (3H, t, *J* 7.4 Hz, **C**H_3-CH_2)**;** δ_C 172.7, 146.7, 146.5, 134.5, 129.6, 127.9, 106.7, 104.0, 101.0, 80.2, 72.7, 62.9, 61.3, 60.8, 56.6, 49.3, 36.5, 28.5, 18.4, 13.8 ppm; ν_{max} 3020.39, 2935.49, 2400.76, 1729.99, 1483.49, 1215.72, 755.48 cm^{-1}; **MS(CI)** 372.2 (100%, $[M+H^+]$) 743.4 (15%, $[2M+H^+]$); **HRMS(ES)** found 372.1801, $C_{21}H_{26}O_5N$ ($[M+H^+]$) requires 372.1805

(3*S*,4a*S*,5*S*,12*R*)-3-methoxy-4,4a-dihydro-3*H*,6*H*-11b,5-ethano[1,3]dioxolo[4,5-*j*]phenanthridin-12-yl hexanoate



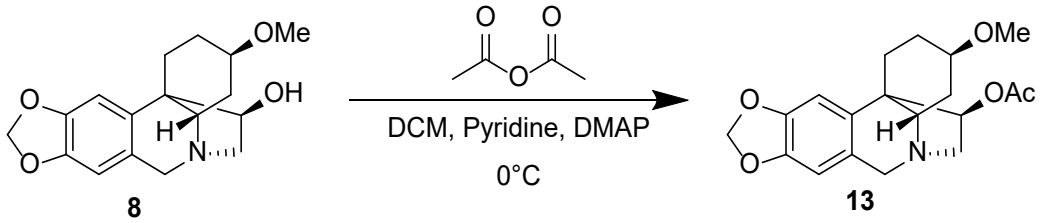
Using method from **4**, **7** (1.0 g, 3.32 mmol) was dissolved in dichloromethane (30 mL), and treated with triethylamine (1.39 mL, 9.96 mmol), DMAP (10 mg) and hexanoyl chloride (0.70 mL, 4.98 mmol). The product was then purified using flash column chromatography (2-5% gradient eluention of MeOH in chloroform) to give **12** (1.146 g, 3.03 mmol, 91% yield) as a gum. Rf 0.73 (10% MeOH in DCM); **δ_H** 6.89 (1H, s, CH), 6.45 (1H, s, CH), 6.33 (1H, d, *J* 10.1 Hz,), 6.13 (1H, dd, *J* 5.0, 10.0 Hz, CH), 5.87 (2H, s, CH2), 4.96 (1H, dd, *J* 3.5, 7.0 Hz, CH), 4.34 (1H, d, *J* 16.8Hz, CH), 3.79-3.84 (1H, m, CH), 3.70 (1H, d, *J* 16.8 Hz, CH), 3.39 (1H, dd, *J* 7.1, 15.4 Hz, CH), 3.34 (3H, s, CH3), 3.29 (1H, dd, *J* 3.5, 14.4, CH), 2.19 (2H, td, *J* 2.7, 7.4 Hz, CH2), 2.03 (1H, dd, *J* 4.3, 13.6 Hz, CH), 2.03 (1H, dd, *J* 4.3, 13.6 Hz, CH), 1.93 (1H, td, *J* 4.2, 12.4 Hz, CH), 1.54 (2H, p, *J* 7.44 Hz, CH2), 1.22-1.33 (4H, m, 2 x CH2), 0.88 (3H, t, *J* 6.8Hz,)**; δ_C** 130.2, 130.1, 129.6, 127.9, 127.5, 127.3, 106.7, 104.1, 104.0, 101.0, 81.1, 80.9, 80.1, 72.7, 63.0, 61.2, 60.7, 56.7, 42.7, 42.4, 34.6, 31.4, 28.4, 24.7, 22.6, 22.5, 14.0 ppm**; ν_{max}** 2957.00, 2871.42, 1736.05, 1483.78, 1239.75, 1091.11, 754.58 cm^{-1}; **MS(CI)** 400.2 (55%, $[M+H^+]$) ; **HRMS(ES)** found 400.2115, $C_{23}H_{30}O_5N$ ($[M+H^+]$) requires 400.2118

(3*R*,4a*S*,5*S*,12*R*)-3-methoxy-2,3,4,4a-tetrahydro-1*H*,6*H*-11b,5-ethano[1,3]dioxolo[4,5-*j*]phenanthridin-12-ol



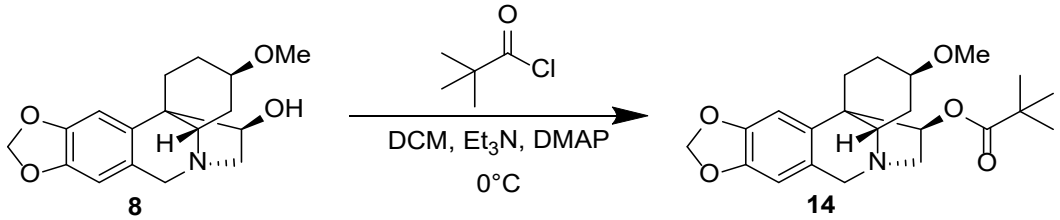
Under a nitrogen atmosphere, palladium on charcoal (10 %, 0.1 g) was added to a solution of haemanthamine **7** (5.00 g, 16.6 mmol) dissolved in dry THF (100 mL). After evacuating the flask, the mixture was stirred at rt under a hydrogen atmosphere for 16 hrs, following which the reaction was filtered through a Celite© pad that was washed with excess THF and the filtrate evaporated under reduced pressure. The residue was purified by flash column chromatography (1-5% MeOH in chloroform, graduated elution) to give dihydrohaemanthamine **8** (3.62 g, 11.9 mmol, 72 % yield) as a colourless crystalline solid. Rf 0.17 (10% MeOH in DCM), **δ_H:** 6.69 (1H, s,), 6.39 (1H, s,), 5.86 (2H, s,), 4.26 (1H, d, *J* 16.70 Hz,), 4.10-4.04 (1H, m,), 3.69 (1H, s,), 3.64 (1H, d, *J* 16.71 Hz,), 3.35-3.19 (7H, m,), 2.34-1.75 (6H, m,); **δ_C** 146.6, 146.0, 139.6, 126.0, 106.2, 103.7, 100.8, 82.4, 75.8, 63.1, 62.8, 60.9, 55.8, 50.7, 46.2, 30.2, 27.0, 22.9 ppm; **MS(CI)** 304.1 (100%, [M+H$^+$]), 607.3 (5%, [2M+H$^+$]); **HRMS(ES)** found 304.1540, $C_{17}H_{22}O_5N$ ([M+H$^+$]) requires 304.1543

(3*R*,4a*S*,5*S*,12*R*)-3-methoxy-2,3,4,4a-tetrahydro-1*H*,6*H*-11b,5-ethano[1,3]dioxolo[4,5-*j*]phenanthridin-12-yl acetate



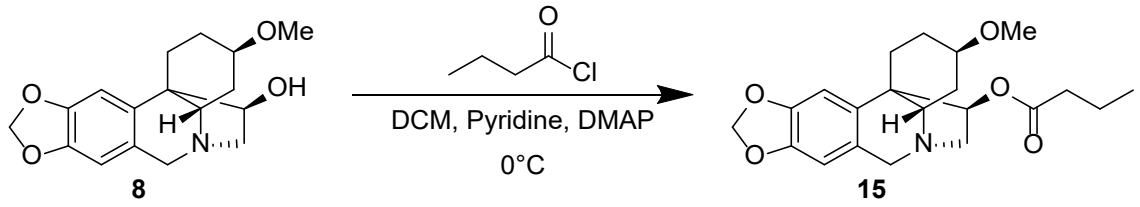
Using the method from **4**, dihydrohaemanthamine **8** (634 mg, 2.09 mmol) was dissolved in dichloromethane (30 mL) and pyridine (0.84 mL, 10.45 mmol), DMAP (10 mg) and acetic anhydride (0.59 mL, 6.27 mmol) were added. The product was then purified using flash column chromatography (1-5% MeOH in chloroform, gradient elution) to give **13** (354 mg, 1.02 mmol, 49 % yield) as a gum. Rf 0.44 (10% MeOH in DCM); δ_H 6.76 (1H, s, CH), 6.56 (1H, s, CH), 5.94 (2H, d, *J* 3.0 Hz, CH2), 4.81 (1H, dd, *J* 3.6, 7.5 Hz, CH), 4.13 (1H, d, *J* 17.0 Hz, CH), 3.66 (1H, s, CH), 3.61 (1H, d, *J* 17.1 Hz, CH), 3.33 (4H, s, 2 x CH2), 3.23-3.31 (1H, m, CH), 3.20 (2H, s, CH2), 2.98 (2H, m, CH2), 2.19-2.33 (1H, m, CH), 1.79-1.93 (4H, m, 2 x CH2), 1.53 (1H, td, *J* 1.2, 14.4 Hz, CH); δ_C 106.0, 103.9, 100.8, 82.5, 75.3, 62.8, 61.2, 60.9, 55.7, 30.3, 26.7, 22.5, 21.3 ppm**;** ν_{max} 3020.21, 1215.69, 756.27 cm^{-1}; **MS(CI)** 346.2 (100%, $[M+H^+]$), 691.3 (5% $[2M+H^+]$) ; **HRMS(ES)** found 346.1641, $C_{19}H_{24}O_5N$ ($[M+H^+]$) requires 346.1649

(3*R*,4a*S*,5*S*,12*R*)-3-methoxy-2,3,4,4a-tetrahydro-1*H*,6*H*-11b,5-ethano[1,3]dioxolo[4,5-*j*]phenanthridin-12-yl pivalate



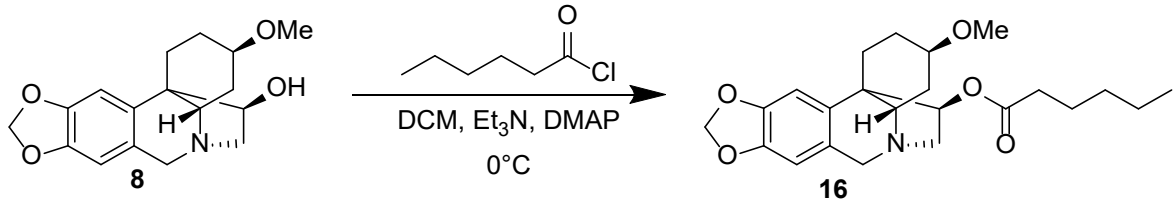
Using the method from **4**, **8** (648 mg, 2.14 mmol) was dissolved in dichloromethane (30 mL) and pyridine (1.49 mL, 10.68 mmol), DMAP (10 mg) and pivaloyl chloride (0.79 mL, 6.41 mmol) were added. The product was then purified using flash column chromatography (1-4% MeOH in chloroform, gradient elution) to give **14** (554 mg, 1.31 mmol, 61 % yield) as a gum. Rf 0.51 (10% MeOH in DCM); δ_H 6.73 (1H, s, CH), 6.41 (1H, s, CH), 5.87 (2H, s, CH2), 4.88 (1H, dd, *J* 3.2, 6.7 Hz, CH), 4.31 (1H, d, *J* 16.8 Hz, CH), 3.73 (1H, s, CH), 3.67 (1H, d, *J* 15.9 Hz, CH), 3.41 (1H, dd, *J* 7.0, 14.5 Hz,), 3.29 (3H, s, CH3), 3.05 (1H, dd, *J* 3.4, 14.5, CH), 2.32-2.29 (1H, m, CH), 1.98-2.13 (4H, m, 2 x CH2), 1.57 (1H, t, *J* 13.3 Hz,), 1.19 (9H, s, 3 x CH3); δ_C 106.2, 104.1, 100.9, 83.1, 75.3, 63.0, 26.0, 61.0, 55.9, 30.4, 27.4, 26.8, 22.5 ppm**;** ν_{max} 3020.31, 1215.68, 759.61 cm^{-1}; **MS(CI)** 388.2 (100%, $[M+H^+]$), 775.4 (15%, $[2M+H^+]$); **HRMS(ES)** found 388.2113, $C_{22}H_{30}O_5N$ ($[M+H^+]$) requires 388.2118

(3*R*,4a*S*,5*S*,12*R*)-3-methoxy-2,3,4,4a-tetrahydro-1*H*,6*H*-11b,5-ethano[1,3]dioxolo[4,5-*j*]phenanthridin-12-yl butyrate



Using method from **4**, **8** (649 mg, 2.14 mmol) in dichloromethane (30 mL), pyridine (0.86 mL, 10.70 mmol), DMAP (10 mg), *n*-butyryl chloride (0.63 mL, 6.42 mmol). The product was purified using flash column chromatography (1-4% MeOH in chloroform, gradient elution) to give **15** (232 mg, 0.62 mmol, 29 % yield) as a gum. Rf 0.49 (10% MeOH in DCM); $\boldsymbol{\delta_H}$ **:** 6.71 (1H, s, CH), 6.40 (1H, s, CH), 5.86 (2H, s, CH2), 5.00 (1H, dd, *J* 3.4, 6.9 Hz, CH), 4.28 (1H, d, *J* 16.9 Hz, CH), 3.70 (1H, s, CH), 3.65 (1H, d, *J* 16.8 Hz, CH), 3.37 (1H, dd, *J* 7.1, 14.3 Hz, CH), 3.27 (3H, s, CH3), 3.12 (1H, dd, *J* 3.5, 14.5 Hz, CH), 2.27 (3H, t, *J* 7.3 Hz, **C**H_3-CH_2), 1.80-2.10 (5H, m), 1.56-1.68 (3H, m), 1.24 (1H, t, *J* 7.1 Hz, CH), 0.94 (3H, t, *J* 7.4 Hz, CH3) ; $\boldsymbol{\delta_C}$ 173.0, 146.7, 146.3, 138.4, 126.3, 106.1, 104.0, 100.9, 82.5, 75.3, 62.8, 61.4, 61.0, 45.2, 36.6, 30.4, 26.8, 22.6, 18.5, 13.7 ppm**;** $\boldsymbol{\nu_{max}}$ 3020.43, 2400.86, 1215.71, 757.23, 669.50 cm^{-1}; **MS(CI)** 374.2 (100%, $[M+H^+]$), 747.4 (10%, $[2M+H^+]$); **HRMS(ES)** found 374.1958, $C_{21}H_{28}O_5N$ ($[M+H^+]$) requires 374.1962

(3*R*,4a*S*,5*S*,12*R*)-3-methoxy-2,3,4,4a-tetrahydro-1*H*,6*H*-11b,5-ethano[1,3]dioxolo[4,5-*j*]phenanthridin-12-yl hexanoate



Using method from **4**, **8** (648 mg, 2.14 mmol) in dichloromethane (30 mL), pyridine (0.87 mL, 10.75 mmol), DMAP (10 mg), hexanoyl chloride (0.1 mL, 6.45 mmol). The product was purified using flash column chromatography (1-2% MeOH in chloroform, gradient elution) to give **16** (711 mg, 1.77 mmol, 83 % yield) as a gum. Rf 0.55 (10% MeOH in DCM); δ_H 6.72 (1H, s, CH), 6.41 (1H, s, CH), 5.87 (2H, s, CH2), 5.02 (1H, dd, *J* 3.4, 7.0 Hz, CH), 4.32 (1H, d, *J* 16.8, CH), 3.69 (2H, d, *J* 16.6 Hz, CH2), 3.30-3.40 (2H, m, CH2), 3.27 (3H, s, CH3), 3.20 (1H, dd, *J* 3.4, 8.5 Hz, CH), 2.15-2.30 (4H, m), 1.81-2.15 (4H,m), 1.57-1.65 (4H, m), 1.24-1.36 (6H, m), 0.88 (3H, t, *J* 6.9 Hz, CH3-CH2); δ_C 177.8, 173.1, 146.9, 146.5, 138.0, 125.5, 106.2, 104.0, 101.0, 82.1, 75.1, 62.8, 60.8, 60.3, 55.8, 45.3, 35.2, 34.6, 31.7, 31.3, 29.8, 26.8, 25.1, 24.7, 22.6, 22.4, 14.0 ppm; ν_{max} 3020.17, 2932.60, 1729.13, 1482.45, 1215.65, 757.39, 699.46 cm^{-1}; **MS(CI)** 402.2 (100%, $[M+H^+]$); **HRMS(ES)** found 402.2266, $C_{23}H_{32}O_5N$ ($[M+H^+]$) requires 402.2275

Chapter 2

"Synthesis of zephycandidine A from haemanthamine"

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Introduction

Zephycandidine A (**17**, Figure 5) was first reported in 2016 by Zhan *et* a,l[10] and described as the first naturally occurring imidazo[1,2-f]phenanthridine alkaloid. The alkaloid was isolated from *Zephyranthes candida* a member of *the amaryllidaeciae* family, which encompasses daffodils, snowdrops, lilies, onions, and garlic.

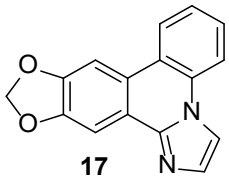
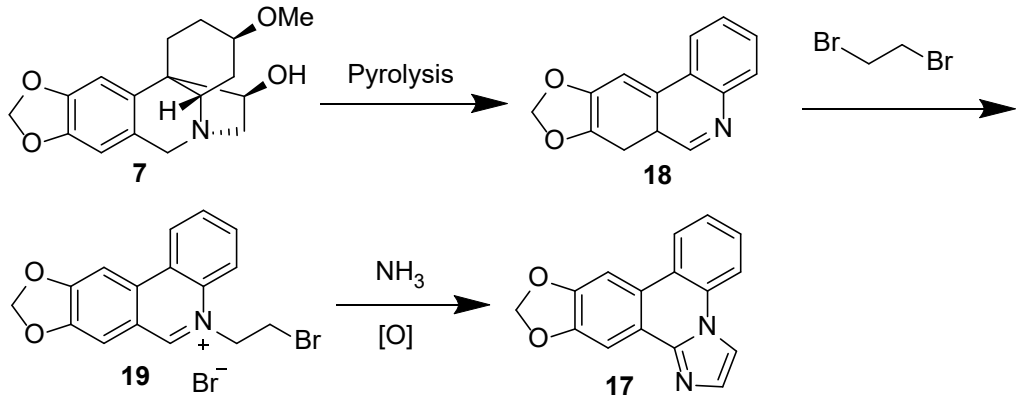


Figure 5: Zephycandidine A

Zhan *et al*. showed that zephycandidine A has some activity against five human cancer cell lines: human myeloid leukemia HL-60, lung cancer A549, breast cancer MCF-7, colon cancer SW480, and hepatocellular carcinoma SMMC-7721. More interestingly, it showed weak cytotoxicity against the normal Beas-2B cell line (IC_{50} = 20.08 µM) with the selectivity indices as high as 10.1.[10]

In the course of our work in the haemanthamine project, the zephycandidine A work was reported and we decided to embark upon a synthesis of the natural product. The paper describes the isolation by extraction and reports an overall yield, from 10 kg of dried plant matter of only 3.5 mg of **17**. We decided at this point to devise a synthetic method in order to access more significant quantities of the compound for potential testing. The authors of the isolation paper proposed a synthesis from the known trispheridine **18** (Scheme 3) might be achieved using a method described by the Cronin group[11]. This would require a synthesis or the isolation of trispheridine **18** and its conversion to the alkylated bromide salt **19** (Scheme 3), which on reaction with ammonia followed by oxidation using MnO_2 should give zephycandidine A **17** (Scheme 3).



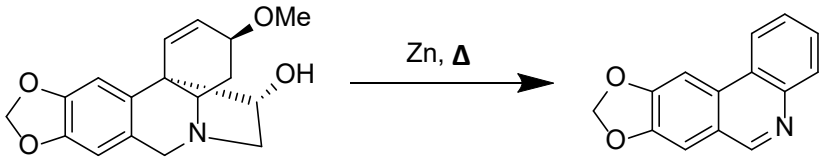
Scheme 3: Proposed synthesis of trispheridine **19**, and zephycandidine A **17**, from haemanthamine **7**.

Several methods for a total syntheses of **17** have been previously suggested[12, 13, 14] but of interest to us was using **18** as a starting material. A report of the preparation of **18** as described by Warren and Wright[15] from haemanthamine **7**. They reported the pyrolysis of haemanthamine **7** on a 0.33 mmol. The paper describes a distillation through a layer of heated zinc powder. Unfortunately, it gives no description of the specific conditions used in the reaction, i.e. the temperature at which the zinc was heated and the relative proportions of zinc to substrate. Due to the lack of details and the inability to contact the original authors, a large portion of time was spent attempting to recreate the experiment at a larger scale in order to prepare a usable quantity of trispheridine **19** for use in the synthesis of Zephycandidine A **17**.

Due to the fact that a large quantity of haemanthamine was available for use in this experiment, a wide array of conditions at gram scale was conducted in order to ascertain the ideal conditions for the pyrolysis of haemanthamine into trispheridine (Scheme 3).

Results and Discussion

As previously stated the preparation of compound **12** was reported by Warren and Wright[15] by pyrolysis of haemanthamine **7** to trispheridine **18** (Scheme 4). However, the paper itself gives very little details as to the conditions under which this pyrolysis is carried out.



7 **18**

Scheme 4: Pyrolysis of **7**

Due to the lack of detailed information in the paper, several large-scale experiments were undertaken using varying conditions (Table 19).

Entry	Scale (mmol)	Temp (°C)	Zn (Equiv)	Time (h)	Yield **18**[i] (%)	Recovered **7** (%)	Decalin (mL)	Sealed
1	16.6	180-90	36	3	10 (24)	59	0	No
2	19.1	190-95	72	24	4 (8)	52	0	No
3	6.6	190-95	185	24	11 (9)	55	0	No
4	16.8	190-95	72	24	11 (22)	50	0	No
5	16.9	210-20	72	7	7	0[ii]	0	No
6	8.3	190-95	75[iii]	24	11(14)	22	0	No
7	8.3	160-90	75[iii]	48	10 (13)	25	0	No
8	1.7	190-95	0	24	**20 (99)**	80	0	Yes
9	5.0	190-95	0[iv]	24	11 (39)	73	0	Yes
10	6.6	175-80	0	120	0	85	2	Yes
11	6.6	180-85	0	168	8 (15)	46	2	Yes
12	6.6	190-95	0	168	19 (22)	14	2	Yes
13	6.6	190-95	0[v]	24	13 (39)	33	2	Yes

Table 19: i) Yields in brackets based on recovered **3**. ii) Considerable decomposition occurred. iii) Using freshly activated zinc. iv) **3** was dispersed on sand. v) 2 equiv. of acetaldehyde added.

Initial experimentation was to determine temperature ranges at which the reaction would occur, during this line of experimentation it was discovered that the temperature must be between 180-210°C, as lower temperatures would give no conversion (Table 19, Entry 10) and higher temperatures would cause total decomposition of the starting material (Table 19, Entry 5). With these data to hand, the optimal temperature was determined to be around 190-195°C (Table 19, Entries 8 & 12).

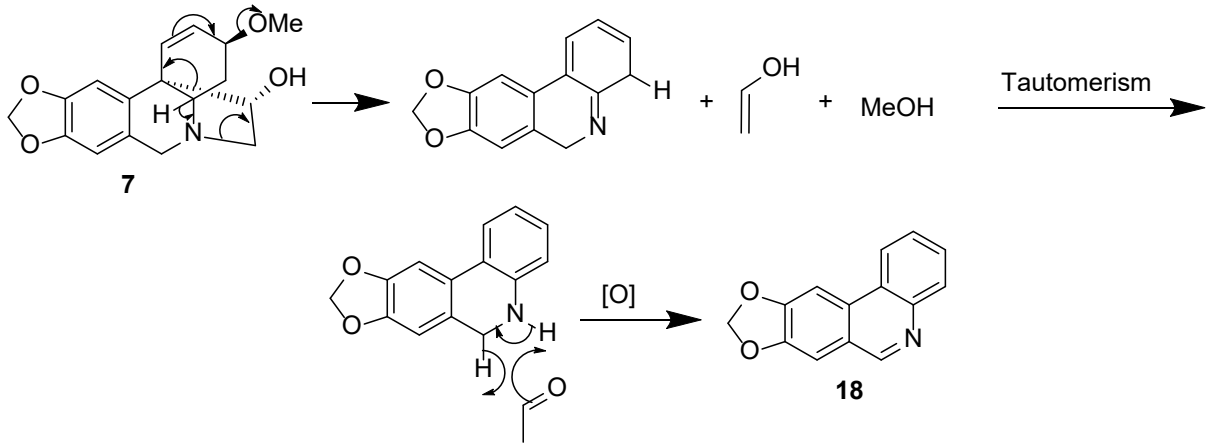
It was mentioned anecdotally that activated zinc may improve the yield of this reaction, activation of the zinc was carried out in the following manner: zinc powder (10 g) was stirred with 1M hydrochloric acid for no more than 2 minutes or until the powder had a

metallic sheen, then the resultant activated zinc powder was suction filtered under nitrogen. This activated zinc was used immediately to minimise oxidation to the surface of the zinc. It was discovered that activation of the zinc gave no improvement in the yield of the reaction, and, as such, this modification was discontinued after 2 attempts (Table 19, Entries 6 & 7).

The paper describes pyrolysis over zinc powder which is a curious approach as the overall reaction involves oxidation, of the substrate and zinc metal is traditionally associated with reductive processes. We thus attempted a range of experiments not involving zinc powder and discovered it was entirely unnecessary for the reaction. Indeed, the removal of the zinc from the reaction gave similar yields as the experiments employing zinc. (Table 19, entries 8-13) It was speculated that dispersion of the starting material on an inert substance might improve the yield of the process, and this might have been the role of the zinc in the original research. We had observed that much of the decomposition observed in the zinc free experiments appeared to be due to aggregation of the starting material (clumping) at the bottom of the reaction flask, which might be leading to burning at the base of the reaction vessel. Sand was chosen, because it was deemed to be unreactive and cheap. As with the previous attempts, the addition of the sand made little to no effect on the overall yield (Table 19, Entry 9). However, it did reduce the amount of decomposition to some extent as shown by the increase in recovered starting material. It was thought that the decrease in decomposition was due to the sand acting as a barrier to localised overheating, giving less opportunities for the starting material to burn with direct contact to the heat. It was also theorised that the reason for significant decomposition and the lack of conversion could be from inconsistency in the heating and mixing of the mixture. We thus looked to a solvent we might use in the reaction, and we used decalin due to its high boiling point, being approximately the same as the observed initiation temperature for the reaction. While the reaction did work in decalin it did not alleviate the overall problems of the reaction or increase the yields. However, reaction 10 gave a good return of starting material at 85%; whereas entry 12 gave return of the starting material at only 14% (Table 19, Entries 10-13).

A mechanism was proposed for the reaction and it was speculated that there is an initial fragmentation step in which a molecule of acetaldehyde and a molecule of methanol are formed. After tautomerization, this results in the formation of the dihydropyridine

derivative, which undergoes subsequent oxidation to give trispheridine **18** (Scheme 5). The source of the oxidant in this process is not known but it may be acetaldehyde as shown or another source in the reaction such as surface zinc oxide or solvent/substrate or indeed the starting material itself. We attempted to achieve this oxidation by the addition of excess acetaldehyde in the reaction mix, however this proved fruitless (Table 19, Entry 13).



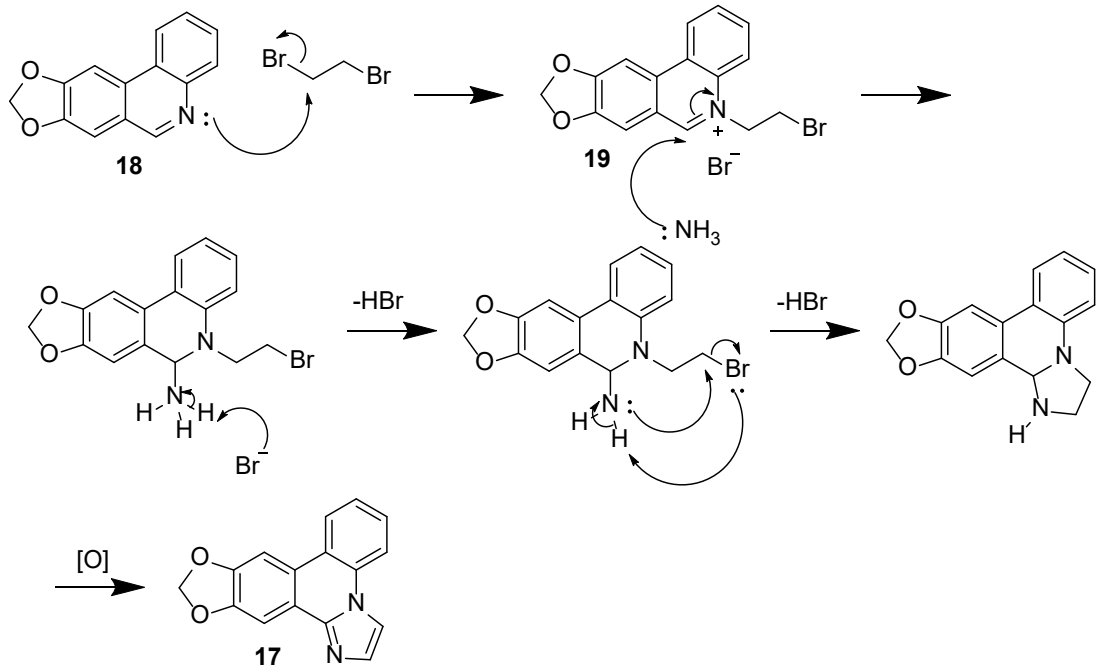
Scheme 5: Proposed mechanism for the preparation of **18** from **7.**

We next moved onto the synthesis of **19** using an adapted method from Cronin *et al*.[11] The synthesis was straightforward and involved heating **18** in 1,2-dibromoethane, collecting the precipitate and washing it with acetone. On analysis of the crude product, it was found that it contained an impurity that was tentatively identified as the hydrobromide of the starting material. This was thought to have arisen by base-catalysed elimination of HBr from the solvent and this by-product proved almost impossible to remove entirely from the product. As a result, the product of this reaction was used without further purification in the next step.

We were able to purify a small quantity of compound **19** by washing a small sample (ca 50 mg) of the impure product with CD_3OD in small portions (ca 1 mL) into which the impurity was slightly more soluble than the product. After two washes the remainder was dissolved in a third and fourth volume of solvent and these were shown (by NMR) to be of >95% purity.

The final step of this reaction involved a 3-step one pot sequence. Firstly, a sample of **19** was dissolved in liquid ammonia at -70 °C, the mixture was brought to reflux (-33 °C) for

an hour. This mixture was then re-cooled to -70 °C and manganese (IV) oxide and sodium carbonate were added. The mixture was then brought back to room temperature slowly, and the ammonia was allowed to evaporate overnight. Finally, toluene was added, and the mixture was then heated to reflux for 3 hours. After filtration and evaporated, purification by column chromatography gave zephycandidine A **17** in a 54% yield.



Scheme 6: Mechanisms for the reactions utilised in the synthesis of zephycandidine A

Synthetic zephycandidine A **17** gave NMR data that were in close agreement to data described in the literature when obtained at 400 MHz. The data were obtained in CD_3OD as in the literature; however, the chemical shifts showed significant variation on allowing the solution to stand and reanalysis. It was postulated that this was due to minor changes in pH of the sample. In addition, it was postulated that, as **17** is only sparingly soluble in CD_3OD, it was thought that, without strict control of the concentration and temperature, variation in the chemical shifts would occur. Reanalysis at 700 MHz was undertaken and the assignments are given in Table 20.

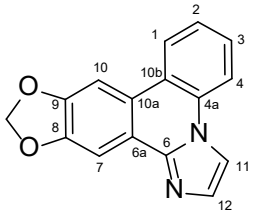


Figure 6: Zephycandidine A, with atom labels.

Position	^{13}C/ppm	^{1}H/ppm	*J*/Hz	Comments
1	125.21	8.39	dddd, 8.1, 1.2, 0.6, 0.6	Couplings to protons at C-2, C-3, C-4 and C-10
2	126.6	7.53	ddd, 8.1; 7.2; 1.2	
3	129.6	7.64	ddd, 8.2; 7.2; 1.2	
4	117.3	8.09	dd, 8.2; 1.2, 0.6	NOESY correlates protons at C-4 and C-11
4a	132.1			
6	143.6			
6a	119.5			HMBC correlates carbon at C-6a to proton at C-10
7	102.9	7.81	d, 0.6	
8	150.5			
9	151.4			
10	102.8	7.90	dd, 0.6, 0.6	
10a	125.23			
10b	122.9			
11	113.7	8.28	d, 1.5	NOESY correlates protons at C-11 and C-4
12	131.2	7.50	d, 1.5	
CH_2	103.5	6.15	d, 0.5	This residual dipolar coupling is a result of some molecules of **1** having preferential orientation in solution at 16.44 T and it has been documented for aromatic molecules before.[16]

Table 20: NMR data for zephycandidine A **17**.

Conclusions

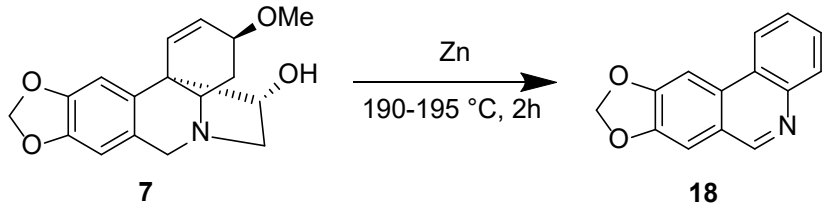
The synthesis of zephycandidine A **17** was achieved in a three-pot, 5 step process (pyrolysis, alkylation, amination, cyclisation, and oxidation) in 10.8% overall yield from the alkaloid haemanthamine **7**. There were some problems with the process, not least of these being the pyrolysis of haemanthamine to give trispheridine **18**. The literature report of this transformation was lacking in precise detail, and it was hard to determine the actual yield reported and the nature of the reagents and apparatus used in the preparation. In writing this report we were struck by the possibility that the zinc we used may have been of too high a purity as an oxidant is needed to affect the final oxidation to give trispheridine **18**. This led us to conclude that repeating the reaction in the presence of zinc oxide might have been an interesting experiment to perform. Despite this we achieved the first semi-synthesis of **17** and, as trispheridine has been the topic of several total syntheses, this work represents the first formal total synthesis.

Experimental

General experimental synthesis and spectroscopy procedures

Reactions were stirred using a magnetic stirrer bar and monitored by TLC on Kieselgel 60 F254 silica gel coated glass plates, which were visualised by either I_2, UV light or phosphomolybdic acid. General chemicals were purchased from Sigma Aldrich, Alfa Aesar, Fluorochem or Fisher Scientific. Flash chromatography was carried out on Fluorochem Silica gel 60Å (40-63 micron) with an eluting solvent as specified. ^{1}H and ^{13}C NMR spectroscopic analyses were carried out on a Bruker Ultrashield Plus 400 MHz spectrometer in $CDCl_3$ unless otherwise stated and are reported in ppm referenced to the solvent internal standard at 7.26 and 77.160 ppm respectively. Infrared spectroscopic analyses were acquired on a Bruker Alpha ATR instrument. Mass spectra were recorded on either a Finnigan MAT 900 XLT or a Finnigan MAT 95 XP at the EPSRC National Mass Spectrometry Service Centre in Swansea.

[1,3]dioxolo[4,5-*j*]phenanthridine (trispheridine)



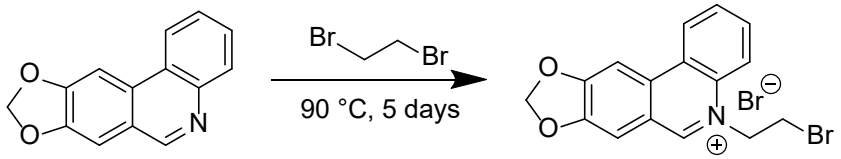
Typical procedure (Table 19, Entry 8): haemanthamine **7**[(i)] (0.50 g, 1.66 mmol) was sealed in a Carius tube and heated at 190-195 °C for 24 h. Purification by column chromatography (80% diethyl ether in hexane) gave **18** (74 mg, 0.33 mmol) as a white solid in 20% yield (99 % based on recovered **7**). Further elution (10 % methanol in chloroform) gave **7** (394 mg, 1.31 mmol, 79 % recovery).

Typical procedure with Zn Dust (Table 19, entry 1): Haemanthamine **7** (5.00 g, 16.6 mmol) was dissolved in methanol (50 mL) and added to finely ground commercial zinc powder (9.00 g, 598 mmol) and the mixture evaporated to dryness under vacuum. This mixture was heated at 190-195 °C for 24 h. Work up as above gave **18** (370 mg, 1.66 mmol) as a white solid in 10% yield (24% based on recovered **1**). Further elution (10 % methanol in chloroform) gave **7** (3.10 g, 10.6 mmol, 62 % recovery).

Typical procedure in decalin (Table 19, Entry 13): Haemanthamine **7** (2.00 g, 6.64 mmol) was added to cis/trans decalin (2 mL), sealed in a Carius tube and the mixture heated at 190-195 °C for 24 h. Work up as above gave **18** (191 mg, 0.857 mmol) as a white solid in 13% yield (39 % based on recovered **7**). Further elution (10 % methanol in chloroform) gave **7** (668 mg, 2.22 mmol, 33 % recovery).

Data for **18** were in agreement with the literature Rf 0.24 (40% EtOAc in PE); Mp 142-145 °C (lit.5e 142.5-144 °C); $\boldsymbol{\delta_H}$ 9.08 (1H, s, CH), 8.36 (1H, br d, J 7.9 Hz, CH), 8.14 (1H, br d, J 8.1 Hz, CH), 7.89 (1H, s, CH), 7.68 (1H, ddd, J 1.2, 7.0, 8.1 Hz, CH), 7.62 (1H, ddd, J 1.2, 7.0, 7.9 Hz, CH), 7.32 (1H, s, CH), 6.16 (2H, s, CH_2); $\boldsymbol{\delta_C}$ 151.9, 151.6, 148.3, 144.3, 130.4, 130.2, 128.1, 126.8, 124.4, 123.2, 122.1, 105.6, 102.0, 100.1; $\boldsymbol{\nu_{max}}$; 3031, 2960, 1620, 1581, 1528, 1497, 1486, 1462, 1394, 1382, 1293, 1254, 1227, 1198, 1111, 1094, 1034, 971, 938, 882, 847, 828, 785, 753, 718, 706, 673, 612, 544, 473, 432; HRMS(ES) found 224.0707, $C_{14}H_{10}NO_2$ ([M+H+]) requires 224.0706.

5-(2-bromoethyl)-[1,3]dioxolo[4,5-j]phenanthridin-5-ium bromide



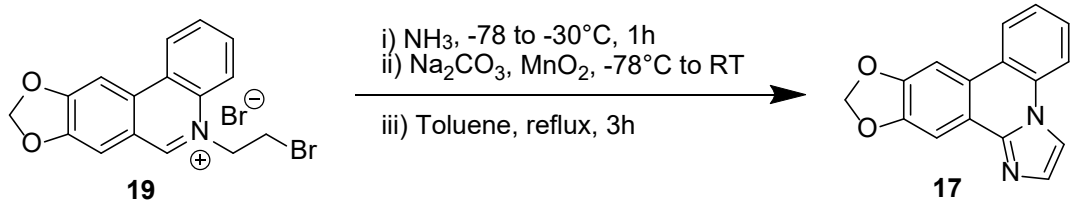
Trispheridine **18** (1.00 g, 4.48 mmol) was suspended in freshly distilled 1,2-dibromoethane (30 mL) and the mixture heated at 70-80 °C for 72 hrs. After cooling, the precipitate formed was removed by filtration and washed with 1,2-dibromoethane (5 mL) and ethyl acetate (2 x 5 mL). After drying under vacuum we obtained the salt 4 (1.56 g) as an off white solid which was contaminated with **2**.HBr (ca 20%). This mixture was used in the next reaction without further purification. An analytical sample was obtained by washing a small sample (ca 20 mg) in a pipette filter sequentially with small portions (5 x 0.6 mL) of CD_3OD to remove **2**.HBr. The 4th and 5th washings contained **19** (ca. 95% pure). Mp 263-266 °C (dec.); $\boldsymbol{\delta_H}$ (CD_3OD) 9.79 (1H, s, CH), 8.99 (1H, dd, J 1.4, 8.3 Hz, CH), 8.48 (1H, s, CH) 8.47 (1H, br d, J 9.2 Hz, CH), 8.11 (1H, ddd, J 1.4, 7.2, 8.5 Hz, CH), 8.04 (1H, ddd, J 1.0, 7.2, 8.3 Hz, CH), 7.83 (1H, s, CH), 6.45 (2H, s, CH_2), 5.46 (2H, t, J 6.0 Hz, CH_2), 4.15 (2H, t, J 6.0 Hz, CH_2); $\boldsymbol{\delta_C}$ (CD_3OD) 159.6, 154.8, 153.0, 152.5, 137.6, 133.3, 132.3, 131.1, 127.3, 126.5, 122.0, 119.8, 108.5, 106.0, 102.3, 59.2, 30.0; $\boldsymbol{\nu_{max}}$; 3476, 3401, 3071, 3021, 2944, 2879, 1653, 1612, 1565, 1538, 1503, 1475, 1427, 1408, 1392, 1353, 1329, 1284, 1257, 1211, 1180, 1154, 1131, 1114, 1036, 1020, 977, 943, 922, 892, 880, 859, 794, 780, 764, 729, 679, 610, 593, 559, 546, 503, 467, 454, 430; HRMS(ES) found 330.0124, $C_{16}H_{14}{}^{79}BrNO_2$ ([M+H+]) requires 330.0124, found 332.0103, $C_{16}H_{14}{}^{81}BrNO_2$ ([M+H+]) requires 330.0104.

Manganese (IV) Oxide[17]

$$2KMnO_4 + 3MnSO_4.H_2O \xrightarrow{60\ °C} 5MnO_2 + K_2SO_4 + 2H_2SO_4$$

A heated (60 °C) solution of manganese sulfate monohydrate (25.00 g, 147.9 mmol) in water (480 mL) was added in portions to a heated (60 °C) solution of potassium permanganate (17.40 g, 110.10 mmol) in water (330 mL). The suspension was stirred at 60 °C for 1h then cooled to room temperature. The solid was suction filtered and washed with water (150 mL) and the solid obtained was dried in an oven (150 °C) for 36h to give activated manganese (IV) oxide (22.40 g, 257.7 mmol, 99.8%) as a black solid.

[1,3]dioxolo[4,5-j]imidazo[1,2-f]phenanthridine



Crude **19** (0.50 g, 1.05 mmol) was added to stirred liquid ammonia (ca 100 mL) at -70 °C and, after removal of the cooling bath, the reaction mixture was warmed to -33 °C. The reaction was kept at this temperature for 1 h then cooled (-78°C) before adding Na_2CO_3 (603 mg; 5.69 mmol) and finely powdered MnO_2 (1.22 g; 14 mmol). After stirring for 1h, the cooling bath was removed to allow the ammonia to evaporate overnight. The residue was dried under vacuum for 10 minutes to remove any remaining ammonia, and then toluene (50 mL) was added. The reaction mixture was heated to reflux for 3 h, cooled and filtered with the solid inorganic residue being washed with acetone (3 x 10 mL). Concentration of the filtrates followed by column chromatography (30-50 % EtOAc in PE) gave **17** (0.15 g, 54 %) as an off-white solid. Recovered trispheridine **18** (0.07 g) was also obtained. Rf 0.29 (50% EtOAc in PE); Mp 242-245 °C (dec.); δ_H (CD_3OD) 8.39 (1H, dd, J 1.2, 8.1 Hz, CH), 8.28 (1H, d, J 1.5 Hz, CH), 8.09 (1H, dd, J 1.2, 8.2 Hz, CH), 7.90 (1H, s, CH), 7.81 (1H, s, CH), 7.64 (1H, ddd, J 1.2, 7.2, 8.2 Hz, CH), 7.53 (1H, ddd, J 1.2, 7.2, 8.1 Hz, CH), 7.50 (1H, d, J 1.5 Hz, CH), 6.15 (2H, s, CH_2); δ_C (CD_3OD) 151.4, 150.5, 143.6, 132.1, 131.2, 129.6, 126.6, 125.23, 125.21, 122.9, 119.5, 117.3, 113.7, 102.9, 103.5, 102.8; δ_H ($CDCl_3/CD_3OD$, 10:1) 8.14 (1H, dd, J 0.8, 8.2 Hz, CH), 7.89 (1H, s, CH), 7.88 (1H, d, J 1.4 Hz, CH), 7.77 (1H, dd, J 0.8, 8.2 Hz, CH), 7.62 (1H, s, CH), 7.51 (1H, ddd, J 1.2, 7.2, 8.2 Hz, CH), 7.48 (1H, d, J 1.4 Hz, CH), 7.42 (1H, ddd, J 1.2, 7.2, 8.2 Hz, CH), 6.08 (2H, s, CH_2); δ_C ($CDCl_3/CD_3OD$, 10:1) 149.6, 148.9, 142.5, 130.9, 130.9, 128.1, 125.2, 123.9, 123.5, 121.7, 119.0, 115.9, 111.7, 102.8, 101.9, 101.4; ν_{max} 3117, 3090, 2917, 2851, 1618, 1536, 1403, 1390, 1313, 1260, 1206, 1174, 1144, 1122, 1034, 945, 929, 905, 848, 827, 768, 736, 692, 681, 620, 584, 472, 444, 423; λ_{max} (MeOH, log ε) 202 (4.62), 228 (4.63), 256 (4.86), 263 (4.89), 296 (4.37) nm; HRMS(ES) found 263.0818, $C_{16}H_{11}N_2O_2$ ([M+H+]) requires 263.0815.

Chapter 3

"Quorum sensing *N*-Acyl homoserine lactones are a new class of anti-schistosomal"

Whiteland. H, Crusco. A, Bloemberg. LW, Tibble-Howlings. J, Forde-Thomas. J, Coghlan. A, Murphy. P. J and Hoffmann. KF.

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Introduction

Schistosoma mansoni is a parasitic flatworm that causes the tropical disease schistosomiasis, also known as snail fever. The disease causes blockage of the digestive and renal system, this causes severe abdominal pain, diarrhoea, and eventually liver and kidney damage, amongst other symptoms. Schistosomiasis is identified by the WHO as the second most socioeconomically impactful parasitic disease to malaria[18,19]. Schistosomiasis is transmitted by contact with water contaminated with the parasitic worms, the water systems becoming contaminated by freshwater snails that are infected with the parasite. Schistosomiasis is found in developing countries with tropical conditions, mostly found in African and Asian countries, but is also common in South America. The disease is especially common in children, as they are most likely to play in contaminated water, but also affects farmers and fishermen who are continually in contact with water systems that may be infected. Approximately 238 million people were infected with schistosomiasis in 2010, with over 85% of the infected being from Africa[20]. The disease predominantly affects children under the age of 14, and in 2010 over 12,000 people died as a direct result of the disease[21], usually due to renal failure, as such this disease is one of the most deadly of neglected tropical diseases.

Currently treatment for the disease is restricted to two anthelmintics: praziquantel **20** and oxamniquine **21** (Figure 7). However some of the side effects of oxamniquine are quite extreme and, as such it is used sparingly especially in pregnant women. Prazquantel **20** is given to patients to treat a number of parasitic worm infections such as schistosomiasis, clonorchiasis, opisthorchiasis, tapeworm infections, cysticercosis, hydatid disease, and other fluke infections[22]. Auranofin **22** is used as an antirheumatic agent[23], meaning it is used to treat the swelling of joints caused by rheumatoid arthritis. It has shown great effectiveness as a vermicide, and is used as a standard as “it completely kills the parasite”[24].

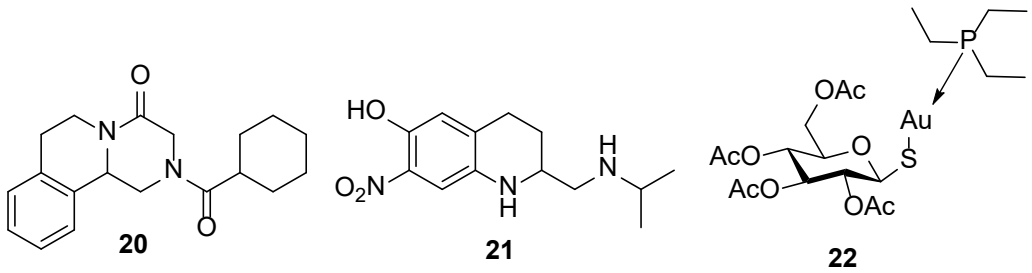


Figure 7: praziquantel **20**, oxamniquine **21** and auranofin **22**.

As part of a random trial, several synthetic compounds were tested for anthelmintic properties (Figure 8). With regards to the effectiveness against the specific phenotype and the motility of *Schistosoma mansoni* parasite, Auranofin (AUR) and Praziquantel (PZQ) is shown below (Figure 8). In these trials DMSO was used as a reference standard. Any compounds that sit within the dashed box are active against this specific trematode. The graph shows how each compound affects the ability of the parasite to move (motility) and how effective it is at targeting sets of characteristics specific to this parasite (phenotype).

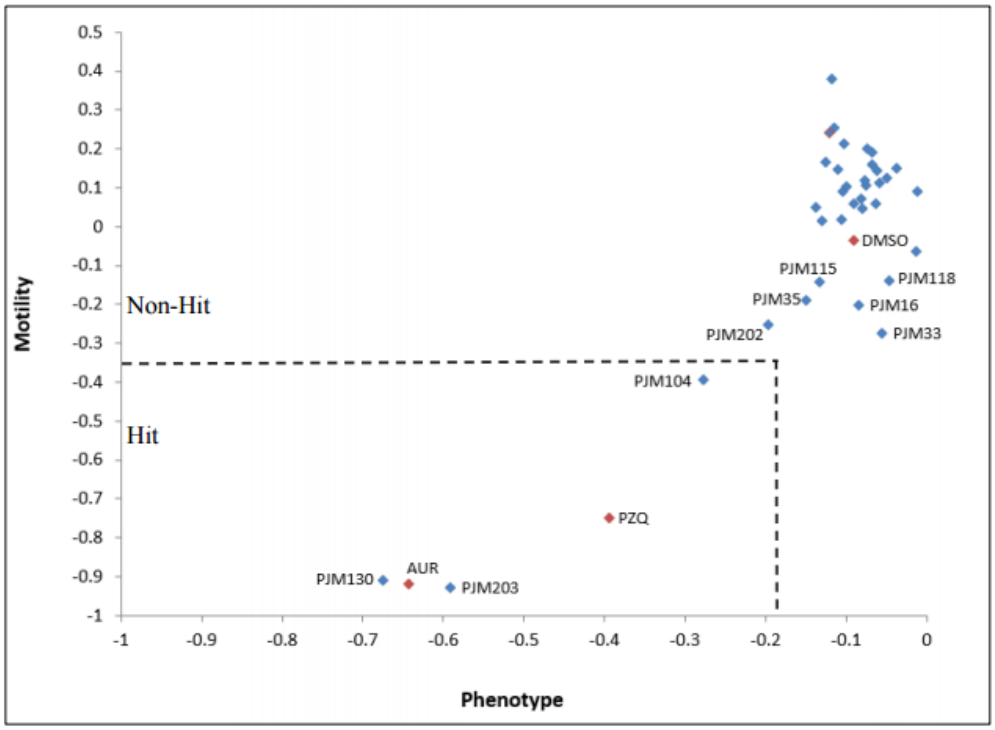
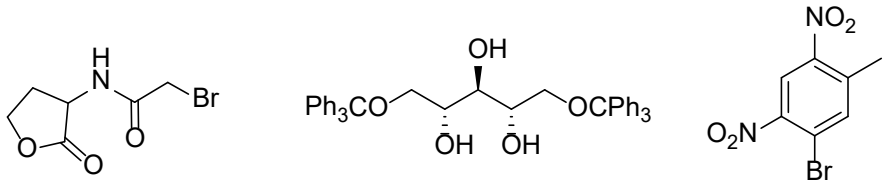


Figure 8: Effects of previously synthesised compounds against phenotype and motility of *Schistosoma mansoni* versus DMSO, Auranofin (AUR) and Praziquantel (PZQ).

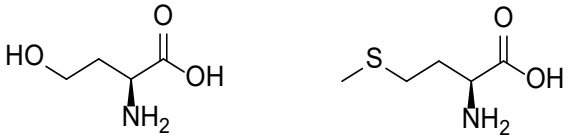
From this it was determined that compounds PJM130 **23** and PJM203 **24** were more effective against the parasite both in respect to their phenotype and motility, than praziquantel **20**. **23** was chosen for derivatisation, this being due in part to the fact that modifications of PJM203 **24** would have been more difficult.



PJM103 **23** PJM203 **24** PJM104 **25**

Figure 9: Compounds **23, 24** and **25.**

The lactone **23** is a derivative of homoserine lactone, which is racemic in the case of **23** but can be derived from the naturally occurring amino acid L-homoserine **26** (Figure 10), which is not one of the common amino acids encoded by human DNA. L-Homoserine is the product of the cleavage of the sulfide group of methionine **27** (Figure 10), which is one of the essential amino acids in humans and is very abundant in animals and plants alike.



26 **27**

Figure 10: L-Homoserine **26** and methionine **27**

It is known that *N*-acyl homoserine lactones (AHLs) (Figure 11) are members of a class of signalling compounds that are used in bacterial quorum sensing (QS)[25]. Quorum sensing is a form of stimulus response to detect population density and control the expression of specific genes. Bacteria use QS to coordinate behaviours such as biofilm formation, virulence, and antibiotic resistance. These are all dependent on the population density around the organism.

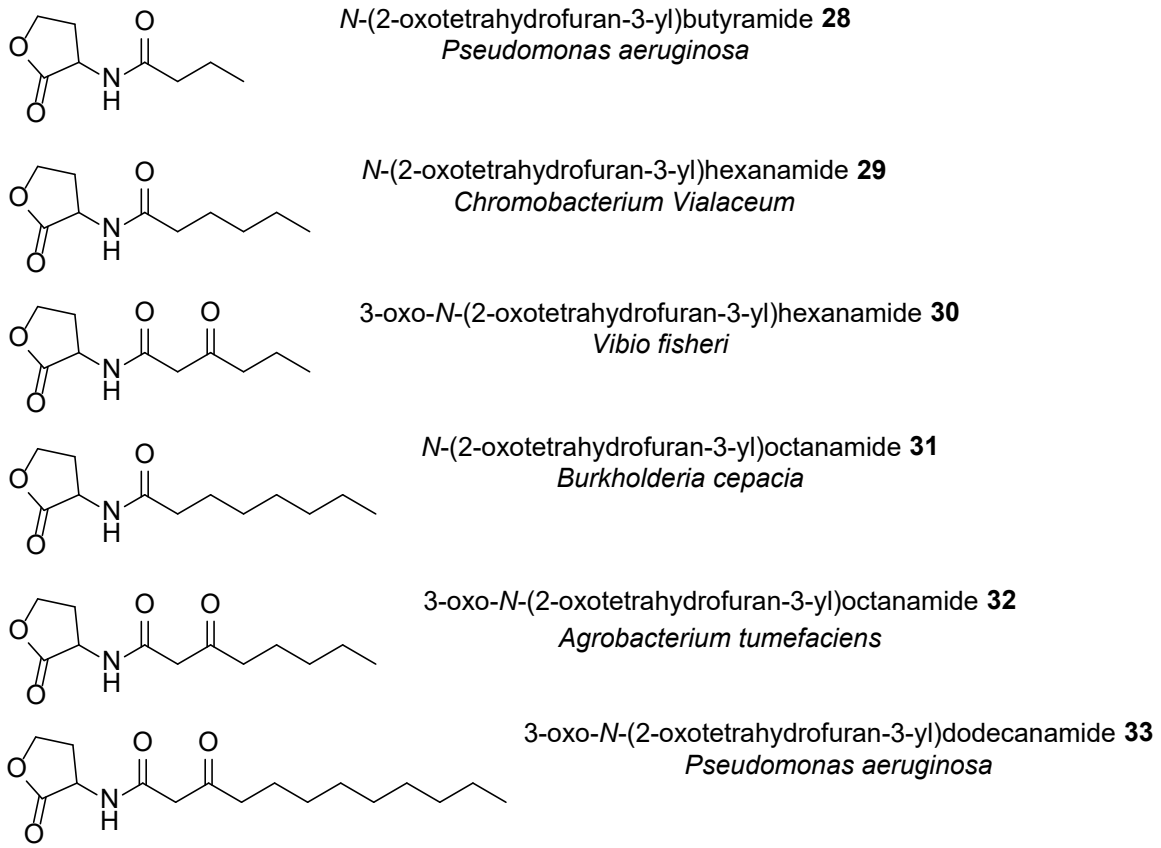
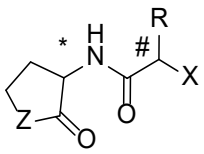


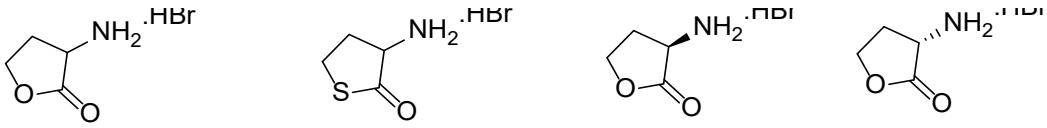
Figure 11: Naturally occurring *N*-acyl homoserine lactones (AHLs) and example species that use this compound for quorum sensing.

We were interested in a general structure **37** that resembles the lead compound **23** as there is considerable scope for relatively straightforward modification of the structure. Modifications are possible at the lactone where X could be O or S, at the halogen where X can be Br or Cl, at the R group or at the two stererogenic centres (* and #).



37

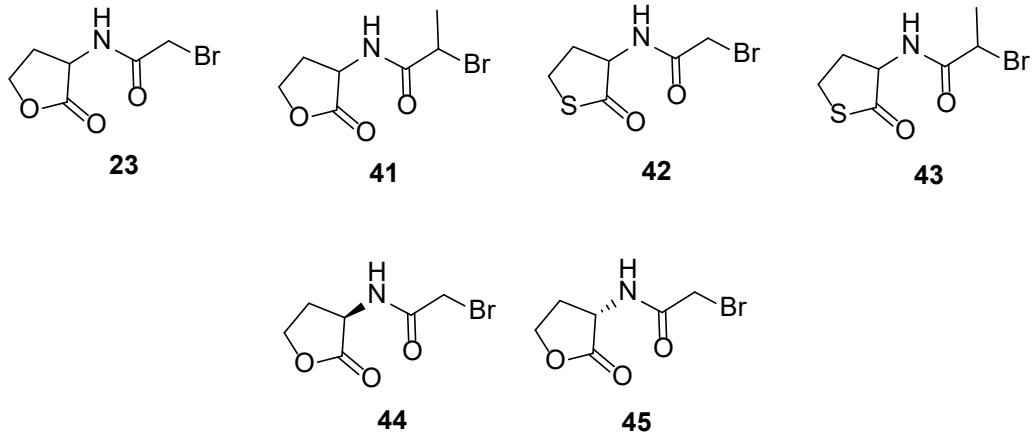
As can be seen the difference between naturally occurring AHLs and synthetic examples are the halogenation and the side-chain, which is typically shorter at either 2 or 3 carbons long. Our precursors for the synthesis are the readily available homoserine lactone hydrobromide **35**, homoserine thiolactone hydrobromide **36**, *S*-homoserine lactone hydrobromide **38** and *R*-homoserine lactone hydrobromide **39**. (Figure 12)



35 **36** **38** **39**

Figure 12: homoserine lactone hydrobromide **35**, homoserine thiolactone hydrobromide **36**, *S*-homoserine lactone hydrobromide **38** and *R*-homoserine lactone hydrobromide **39**.

Derivatisation of these with bromoacetyl bromide, chlororacetyl chloride, bromopropionyl bromide or chloropropionyl chloride will lead to the preparation of a range of compounds **23** & **41-49** (Figure 13), which will enable the investigation of the relationship between the structure of the lactone, the side-chain, and the halogen on the side-chain against the *Schistosoma mansoni* (flatworm) parasite.



23 **41** **42** **43**

44 **45**

46 **47** **48** **49**

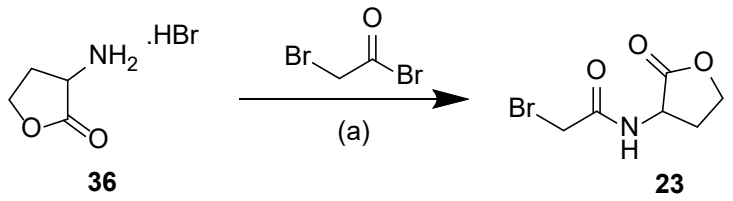
Figure 13: Proposed synthetic AHLs **23** & **41-49**

Results and Discussion

All of the compounds **23** & **41**-**49** were prepared by either a single phase or two-phase protocol, Methods A and B respectively. The two-phase protocol was originally chosen for its simplicity and use of less aggressive reagents. However, there were some problems with the two-phase protocol, in that it gave very poor yields in two of the experiments detailed below. For this reason, a single-phase protocol was adopted and resulted in higher yields. Purification was the same for both methods, flash chromatography (50% EtOAc in chloroform) and gave the desired compounds in high purity

Compound 23

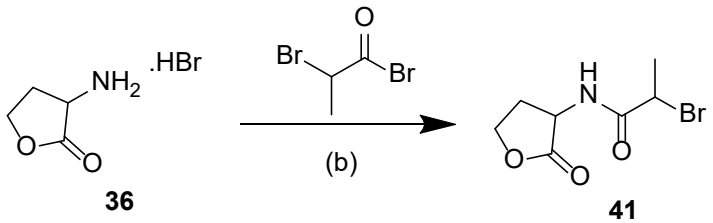
Compound **23** was prepared via method A, giving a yield of 75%. This reaction gave the best yield of all the experiments and the crude product was easy to purify, as were all of the other compounds. These reaction conditions would be considered the best for this transformation; however, a second method was applied to most of the other compounds as it resulted in a simpler workup and purification, albeit resulting in a lower yield overall.



Scheme 7: preparation of **23**. (a) bromoacetyl bromide, $CHCl_3$, NEt_3, 18 h, 75%.

Compound 41

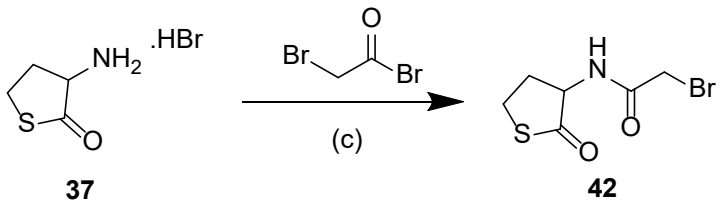
Compound **41** was also prepared using method A in 33% yield. The reason for the poor yield is not known; however, it is an anomalous result amongst the other compounds prepared using method A.



Scheme 8: preparation of **41**. (b) bromopropanoyl bromide, $CHCl_3$, NEt_3, 18 h, 33%.

Compound 42

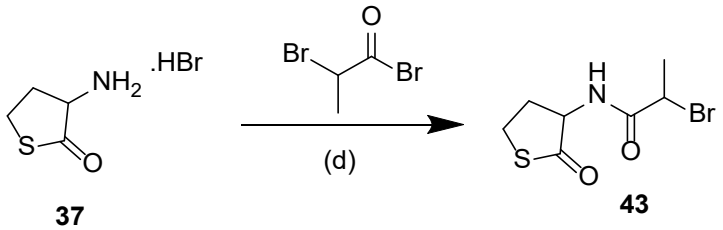
Compound **42** was prepared using the two-phase method B, giving a reasonable yield of 40% yield, again it is unknown as to why this reaction was not more high yielding.



Scheme 9: preparation of **42.** (c) bromoacetyl bromide, CH_2Cl_2 / H_2O, K_2CO_3, 18 h, 40%.

Compound 43

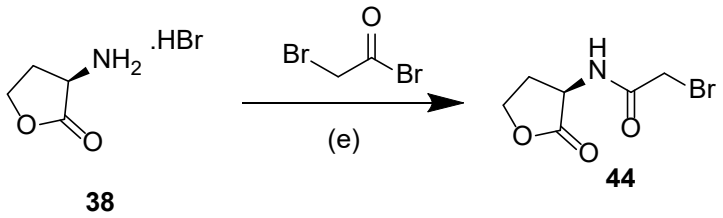
Compound **43** was prepared using method B, resulting in a 73% yield, which was considerably higher yield than that of corresponding lactone **41**.



Scheme 10: preparation of **43.** (d) bromopropanoyl bromide, CH_2Cl_2 / H_2O, K_2CO_3, 18 h, 73%.

Compound 44

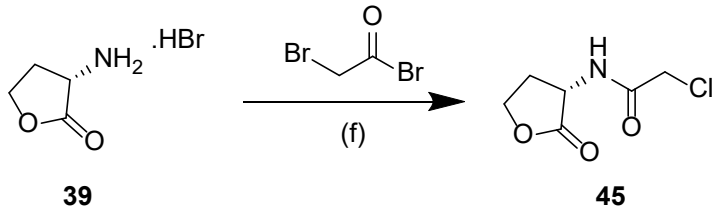
The first of the enantiomerically pure compounds **44** was prepared using the two-phase method B, however this gave a poor 25% yield. The low yield of this reaction, in comparison to the racemic compound **40** was disappointing and may have been due to a change in method.



Scheme 11: preparation of **44** (e) bromoacetyl bromide, CH_2Cl_2 / H_2O, K_2CO_3, 18 h, 25%.

Compound 45

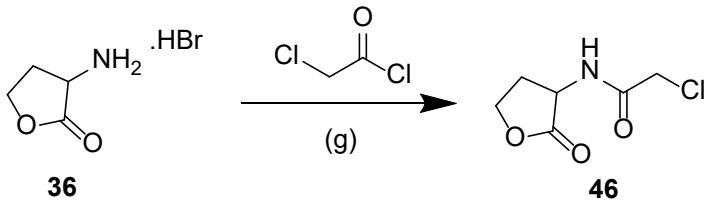
Similarly, compound **45** was prepared using the two-phase protocol and was by far the least efficient reaction, resulting in a yield of only 14%. Again, we are unsure as to the reason for this poor yield and, whilst repeating the reaction using method A would have been interesting, the quantity of sample produced in this, and the previous, reaction was sufficient for testing.



Scheme 12: preparation of **45.** (f) bromoacetyl bromide, CH_2Cl_2 / H_2O, K_2CO_3, 18 h, 14%.

Compound 46

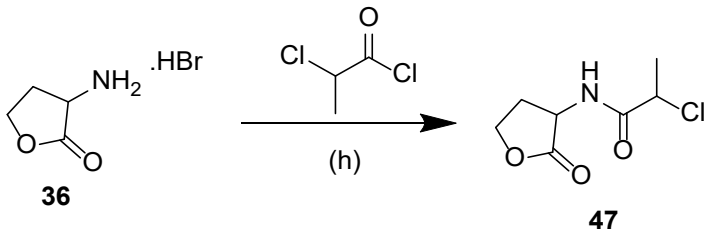
For the chlorinated analogue **46**, method A was readopted, and this gave the desired compound in a considerably improved 68% yield.



Scheme 13: preparation of **46.** (g) chloroacetyl chloride, $CHCl_3$, NEt_3, 18 h, 68%.

Compound 47

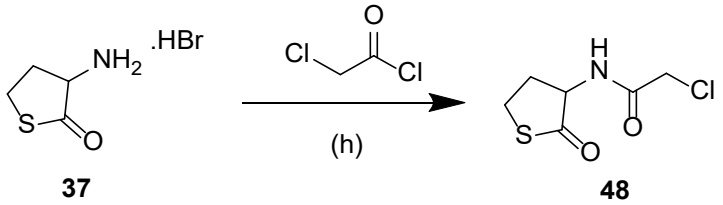
The chloropropionyl amide **47** was also prepared using the single-phase method A, in a respectable 65% yield.



Scheme 14: preparation of **47.** (g) chloropropanoyl chloride, $CHCl_3$, NEt_3, 18 h, 65%.

Compound 48

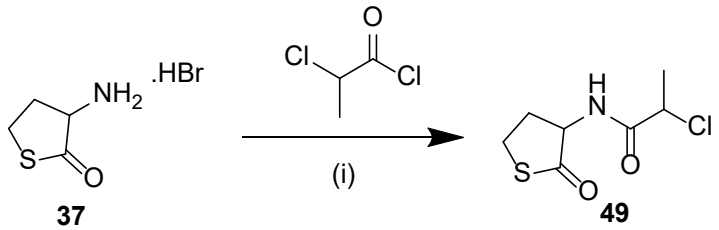
The thiolactone **48** was prepared using the two-phase protocol and, despite the poor yields for the preparation of **44** and **45**, this reaction gave a respectable 61% yield. The reason for the return to the previous method was to confirm a theory that the two-phase method was better for the thiolactone substrates as opposed to the lactones. This result offers some support for this hypothesis.



Scheme 15: preparation of **48.** (h) chloroacetyl chloride, CH_2Cl_2 / H_2O, K_2CO_3, 18 h, 61%.

Compound 49

Similarly, the thiolactone **49** was prepared using the two-phase protocol, which gave the required compound in a 58% yield. Again, a good yield was obtained using method B for a thiolactone.

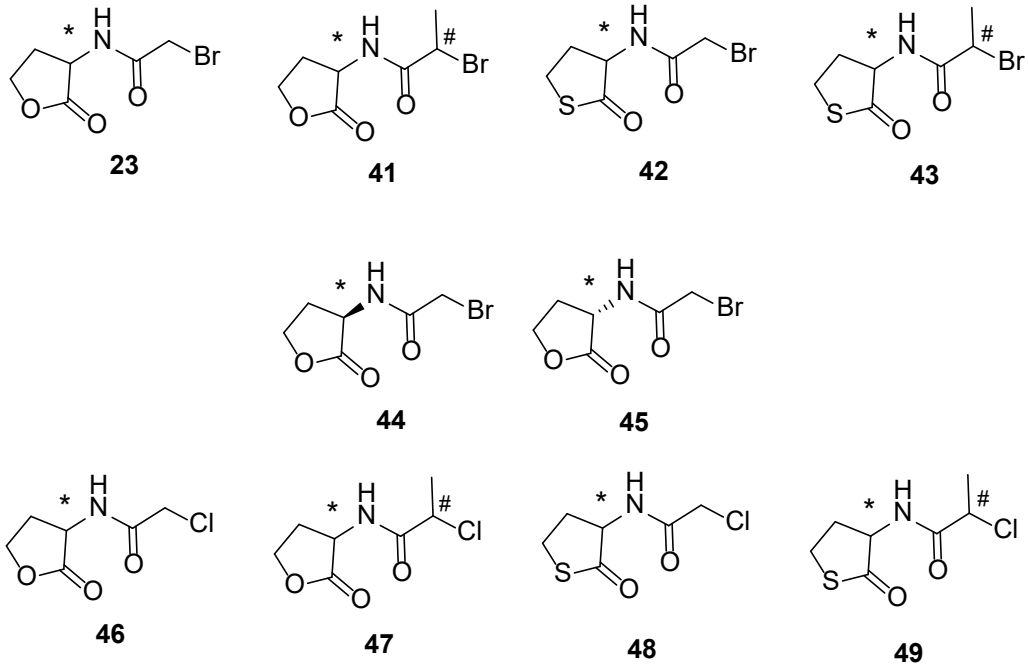


Scheme 16: preparation of **49.** (i) chloropropanoyl chloride, CH_2Cl_2 / H_2O, K_2CO_3, 18 h, 58%.

Conclusions

In conclusion, it is apparent that the preparation of the analogues **40-49** was successful. However, the methods used need to be optimised if any of these compounds are to be of significant need in future work. Specifically, the two-phase method appears to be somewhat capricious. The compounds were all white solids and gave correct analytical data

and were of high purity for the next stage of the project. The structures are summarised below as are the yields and melting point data.



Compound	X	Z	R	*	#	Method[(i)]	Yield	Mp (°C)
23	Br	O	H	Racemic	N/A	A	75%	95-97
41	Br	O	Me	Racemic	Racemic	A	33%	157-169
42	Br	S	H	Racemic	N/A	B	40%	111-113
43	Br	S	Me	Racemic	Racemic	B	73%	125-128
44	Br	O	H	(R)	N/A	B	25%	130-133
45	Br	O	H	(S)	N/A	B	14%	130-133
46	Cl	O	H	Racemic	N/A	A	68%	111-115
47	Cl	O	Me	Racemic	N/A	A	65%	148-150
48	Cl	S	H	Racemic	N/A	B	61%	124-127
49	Cl	S	Me	Racemic	Racemic	B	58%	103-110

(i) The reactions were undertaken in either method A, a single-phase reaction, or method B, a two-phase reaction.

Table 21: Yields of compounds **23** & **41-49** and melting point data.

Biological activity studies

Of the nine compounds tested, four affected the motility of the flatworm (**23**, **42**, **43** and **44**). Compounds **43** and **44** worked at both 10 and 5 µM; whereas compounds **23** and **42** showed effectiveness at all concentrations tested in this study. Analogues **43**, **44** and **46** affected the phenotype of the parasites at 10 and 5 µM; whereas compound **33** affected it from 10 to 1.25 µM and compound **42** was effective at all three concentrations. The modification of the lactone ring (**23**) to a thiolactone ring (**42**) increased the comparative potency at all concentrations. The two enantiomeric forms of compound **23** are of particular interest in this study and it was observed that the (*R*)-enantiomer (**44**) affected schistosomula for both phenotype and motility at 10 µM and 5 µM concentrations. Interesting, no anti-schistosomula activity was observed for the (*S*)-enantiomer (**45**), suggesting that the absolute stereochemistry of the *N*-acyl homoserines is critical to activity.

Experimental

General experimental synthesis and spectroscopy procedures

Reactions were stirred using a magnetic stirrer bar and monitored by TLC on Kieselgel 60 F254 silica gel coated glass plates, which were visualised by either I_2, UV light or phosphomolybdic acid. General chemicals were purchased from Sigma Aldrich, Alfa Aesar, Fluorochem or Fisher Scientific. Flash chromatography was carried out on Fluorochem Silica gel 60Å (40-63 micron) with an eluting solvent as specified. ^{1}H and ^{13}C NMR spectroscopic analyses were carried out on a Bruker Ultrashield Plus 400 MHz spectrometer in $CDCl_3$ unless otherwise stated and are reported in ppm referenced to the solvent internal standard at 7.26 and 77.160 ppm respectively. Infrared spectroscopic analyses were acquired on a Bruker Alpha ATR instrument. Mass spectra were recorded on either a Finnigan MAT 900 XLT or a Finnigan MAT 95 XP at the EPSRC National Mass Spectrometry Service Centre in Swansea. Specific rotations were measured in a 0.25 dm cell on an ADP440 polarimeter (Bellinghan & Stanley Ltd.)

Preparation of lactones 23 & 41-49

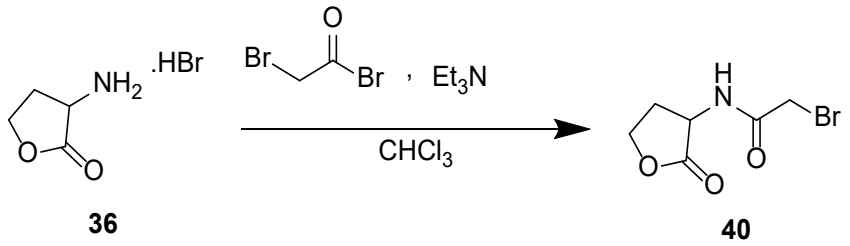
Method A

The acyl halide (1.00-2.65 equiv.) was added dropwise over 5 min to a stirred and cooled (0°C) solution of the aminolactone salt (1 equiv.) and triethylamine (2 equiv.) dissolved in chloroform. After 2 h the reaction was warmed to rt and stirred for 16 h. After evaporation, the mixture was triturated with EtOAc (3 x 10 mL), filtered, evaporated, and purified via column chromatography.

Method B

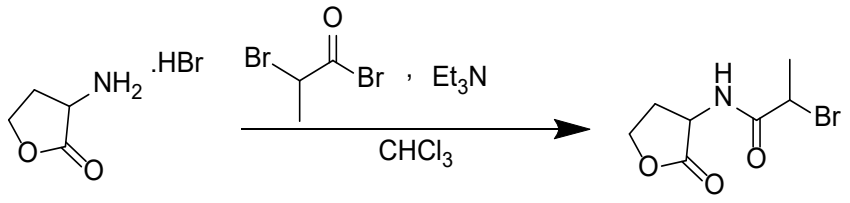
The acyl halide (1.00-2.65 equiv.) was added dropwise over 5 min to a stirred and cooled (0°C) solution of the aminolactone salt (1 equiv.) and potassium carbonate (3. equiv.) in a mixture of chloroform and water. After 2 h the reaction was warmed to rt and stirred for 16 h; whereupon the organic layer was separated, and the aqueous phase extracted with chloroform (20 mL). The combined organic layers were washed with tartaric acid solution (5% aq., 2 x 5 mL) and water (2 x 5 mL), dried over $MgSO_4$, filtered, evaporated, and purified by column chromatography

2-Bromo-*N*-(2-oxotetrahydrofuran-3-yl)acetamide



Method A: Bromoacetyl bromide (1.47 g, 7.27 mmol, 0.63 mL), **36** (500 mg, 2.75 mmol), trimethylamine (556 mg, 5.49 mmol). The reaction mixture was evaporated and purified via column chromatography (50% EtOAc in chloroform) to give **23** (612 mg, 2.76 mmol) in 75% yield as a white solid. $\mathbf{d_H}$ 6.95 (1H, br s, NH), 4.56 (1H, ddd, *J* 6.1, 8.6, 11.5 Hz, CH), 4.52 (1H, br t, *J* 8.9 Hz, CH), 4.33 (1H, ddd, *J* 5.9, 9.5, 11.1 Hz), 3.96 (1H, d, *J* 14.0 Hz, CH), 3.92 (1H, d, *J* 14.0 Hz, CH), 2.84-2.92 (1H, m, CH), 2.18-2.29 (1H, m, 1H) ; $\mathbf{d_C}$ 174.6, 166.4, 66.2, 50.0, 30.1, 28.4; $\boldsymbol{\nu_{max}}$ 3250, 3062, 1760, 1659, 1548, 1180; MS(CI) 222.0 (100%, $[C_6H_8{}^{79}BrNO_3+H]^+$) 224.0 (98%, $[C_6H_8{}^{81}BrNO_3+H]^+$), 244.0 (85%, $[C_6H_8{}^{79}BrNO_3+Na]^+$), 246.0 (85%, $[C_6H_8{}^{79}BrNO_3+Na]^+$); HRMS(ES) found 221.9762, $C_6H_9{}^{79}BrNO_3{}^+$ ($[M+H]^+$) requires 221.9760; Microanalysis: found C 32.6, H 3.8, N 6.4, Br 36.0; $C_6H_8BrNO_3$ requires C 32.4, H 3.6, N 6.3, Br 36.0.

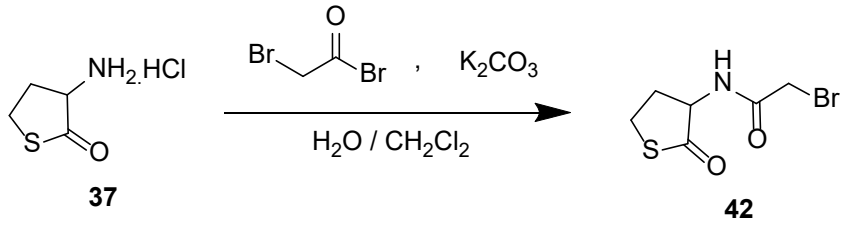
2-Bromo-*N*-(2-oxotetrahydrofuran-3-yl)propanamide



36 **41**

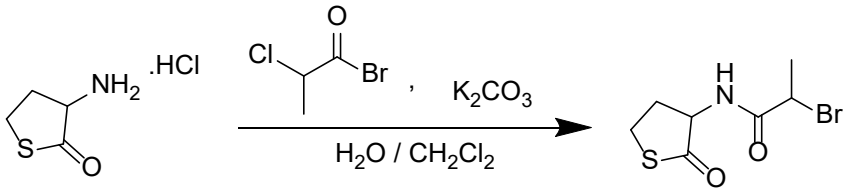
Method A: Bromopropionoyl bromide (593 mg, 2.75 mmol, 0.29 mL), **36** (500 mg, 2.75 mmol), trimethylamine (556 mg, 5.49 mmol). The reaction mixture was evaporated, and the crude was purified via column chromatography (50% EtOAc in chloroform) to give **41** (215 mg, 0.91 mmol) in 33% yield as a white solid. $\mathbf{d_H}$ 6.82/6.77 (1H, 2 br s, NH), 4.41-4.58 (3H, m, 3 x CH), 4.27-4.34 (1H, m, CH), 2.83-2.90 (1H, m, CH), 2.14-2.26 (1H, m, CH), 1.89/1.91 (3H, 2 x d, *J* 6.7 Hz, 2 x CH_3); $\mathbf{d_C}$ 66.2, 50.0/49.9, 43.9/43.7, 30.2/30.2, 22.9/22.8 (2 x C not observed); ν_{max} 3284, 3083, 2946, 1775, 1656, 1551, 1165; MS(CI) 236.0 (80%, $[C_7H_{10}{}^{79}BrNO_3+H]^+$) 238.0 (80%, $[C_7H_{10}{}^{81}BrNO_3+H]^+$), 258.0 (100%, $[C_7H_{10}{}^{79}BrNO_3+Na]^+$), 260.0 (98%, $[C_7H_{10}{}^{81}BrNO_3+Na]^+$); HRMS(ES) found 235.9919, $C_7H_{11}{}^{79}BrNO_3{}^+$ ($[M+H]^+$) requires 235.9917.

2-Bromo-*N*-(2-oxotetrahydrothiophen-3-yl)acetamide



Method B: Bromoacetyl bromide (1.31 g, 6.51 mmol, 0.57 mL), **37** (500 mg, 3.25 mmol). The crude product was purified via column chromatography (50% EtOAc in chloroform) to give **42** (307 mg, 1.29 mmol) in 40% yield as a white solid. $\mathbf{d_H}$ 6.92 (1H, s, NH), 4.51 (1H, ddd, *J* 6.6, 6.6, 12.4 Hz, CH), 3.93 (1H, d, *J* 13.7 Hz, CH), 3.89 (1H, d, *J* 13.7 Hz, CH), 3.38 (1H, ddd, *J* 5.1, 11.6, 12.0 Hz, CH), 3.23 (1H, ddd, *J* 0.8, 7.0, 12.2 Hz, CH), 2.88-2.94 (1H, m, CH), 2.00 (dddd, *J* 7.0, 12.0, 12.2, 12.4 Hz, CH); $\mathbf{d_C}$ 204.6, 166.3, 59.9, 31.5, 28.6, 27.7; $\boldsymbol{\nu_{max}}$ 3262, 1697, 1658, 1537, 1453; MS(CI) 238.0 (95%, $[C_6H_8{}^{79}BrNO_2S+H]^+$) 240.0 (100%, $[C_6H_8{}^{81}BrNO_2S+H]^+$), 260.0 (80%, $[C_6H_8{}^{79}BrNO_2S+Na]^+$), 262.0 (85%, $[C_6H_8{}^{81}BrNO_2S+Na]^+$); HRMS(ES) found 237.9534, $C_6H_9{}^{79}BrNO_2S^+$ ($[M+H]^+$) requires 237.9532.

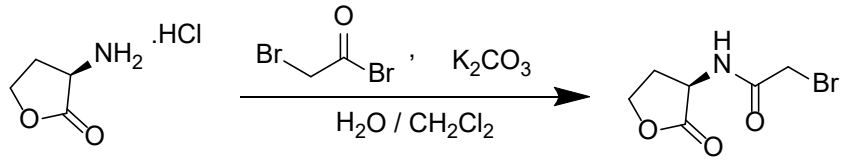
2-Chloro-*N*-(2-oxotetrahydrothiophen-3-yl)propanamide



37 **43**

Method B: Bromopropionyl bromide (1.41 g, 6.51 mmol, 0.68 mL), **37** (500 mg, 3.25 mmol). The crude product was purified via column chromatography (50% EtOAc in chloroform) to give **43** (598 mg, 2.37 mmol) in 73% yield as a white solid. $\mathbf{d_H}$ 6.82/6.76 (1H, 2 x br s, 2 x NH), 4.40-4.53 (1H, m, CH), 3.34-3.41 (1H, m, CH), 3.25-3.30 (1H, m, CH), 2.90-2.96 (1H, m, CH), 1.93-2.04 (1H, m, CH), 1.86/1.89 (3H, d/d, *J* 7.0/7.1 Hz, 2 x CH_3); $\mathbf{d_C}$ 204.9/204.7, 170.1/170.1, 60.0/60.0, 44.2/43.9, 31.6/31.5, 27.7/27.7, 23.0/22.8; $\boldsymbol{\nu_{max}}$ 3256, 3080, 2970, 1686, 1644, 1553; MS(CI) 252.0 (95%, $[C_7H_{10}{}^{79}BrNO_2S+H]^+$) 254.0 (100%, $[C_7H_{10}{}^{81}BrNO_2S+H]^+$), 269.0 (65%, $[C_7H_{10}{}^{79}BrNO_2S+Na]^+$), 271.0 (65%, $[C_7H_{10}{}^{81}BrNO_2S+Na]^+$); HRMS(ES) found 251.9688, $C_7H_{11}{}^{79}BrNO_2S^+$ ($[M+H]^+$) requires 251.9688.

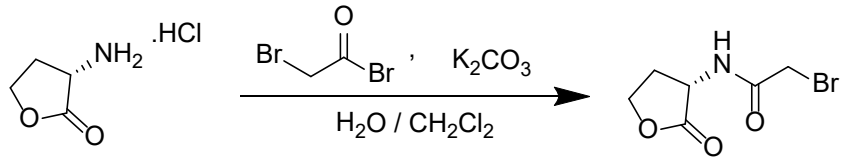
(*R*)-2-Bromo-*N*-(2-oxotetrahydrofuran-3-yl)acetamide



38 **44**

Method B: Bromoacetyl bromide (1.47 g, 7.27 mmol, 0.63 mL), (*R*)-homoserine lactone hydrochloride **38** (500 mg, 3.63 mmol). The crude product was purified via column chromatography (50% EtOAc in chloroform) to give **44** (204 mg, 0.92 mmol) in 25% yield as a white solid. $[\mathbf{d}]_D^{19}$ -22 (c = 0.1 $CHCl_3$), $\mathbf{d_H}$ 6.91 (1H, br s, NH), 4.55 (1H, ddd, *J* 6.1, 8.6, 11.5 Hz, CH), 4.52 (1H, br t, *J* 8.9 Hz, CH), 4.34 (1H, ddd, *J* 5.9, 9.5, 11.1 Hz), 3.96 (1H, d, *J* 13.7 Hz, CH), 3.92 (1H, d, *J* 13.7 Hz, CH), 2.82-2.90 (1H, m, CH), 2.15-2.26 (1H, m, 1H); $\mathbf{d_C}$ 174.6, 166.4, 66.2, 50.0, 30.2, 28.4; $\boldsymbol{\nu_{max}}$ 3253, 3064, 1762, 1657, 1551, 1179; MS(CI) 222.0 (98%, $[C_6H_8{}^{79}BrNO_3+H]^+$) 224.0 (97%, $[C_6H_8{}^{81}BrNO_3+H]^+$), 244.0 (98%, $[C_6H_8{}^{79}BrNO_3+Na]^+$), 246.0 (98%, $[C_6H_8{}^{79}BrNO_3+Na]^+$); HRMS(ES) found 221.9762, $C_6H_9{}^{79}BrNO_3{}^+$ ($[M+H]^+$) requires 221.9760.

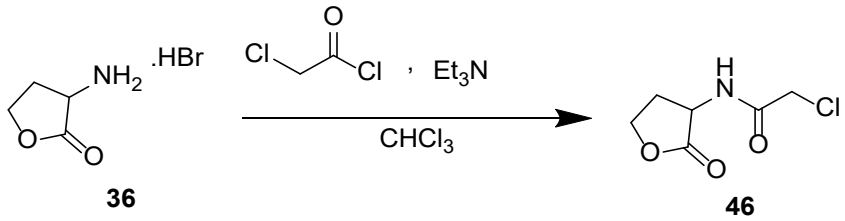
(*S*)-2-Bromo-*N*-(2-oxotetrahydrofuran-3-yl)acetamide[27]



39 **45**

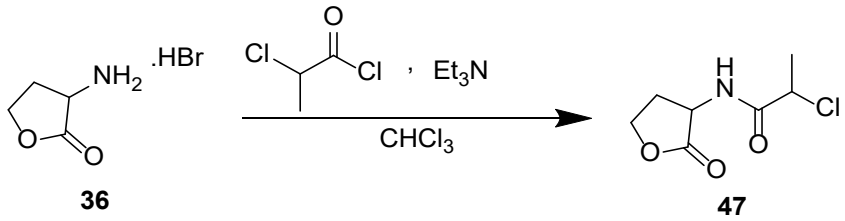
Method B: Bromoacetyl bromide (1.47 g, 7.27 mmol, 0.63 mL), (*S*)-homoserine lactone hydrochloride **39** (500 mg, 3.63 mmol). The crude product was purified via column chromatography (50% EtOAc in chloroform) to give **45** (116 mg, 0.52 mmol) in 14% yield as a white solid. $[\mathbf{d}]_D^{19}$ 21 (c = 0.1 $CHCl_3$), Lit.[26] $[\mathbf{d}]_D^{22}$ 20.5 (c = 0.0074 $CHCl_3$); $\mathbf{d_H}$ 6.99 (1H, s, NH), 4.55 (1H, ddd, *J* 6.1, 8.6, 11.5 Hz, CH), 4.52 (1H, br t, *J* 8.9 Hz, CH), 4.34 (1H, ddd, *J* 5.9, 9.5, 11.1 Hz), 3.96 (1H, d, *J* 13.7 Hz, CH), 3.92 (1H, d, *J* 13.7 Hz, CH), 2.81-2.87 (1H, m, CH), 2.17-2.28 (1H, m, 1H); $\mathbf{d_C}$ 174.7, 166.4, 66.2, 49.9, 30.1, 28.4; $\boldsymbol{\nu_{max}}$ 3254, 3065, 1762, 1656, 1551, 1177; MS(CI) 222.0 (100%, $[C_6H_8{}^{79}BrNO_3+H]^+$) 224.0 (98%, $[C_6H_8{}^{81}BrNO_3+H]^+$), 244.0 (85%, $[C_6H_8{}^{79}BrNO_3+Na]^+$), 246.0 (85%, $[C_6H_8{}^{79}BrNO_3+Na]^+$); HRMS(ES) found 221.9761, $C_6H_9{}^{79}BrNO_3{}^+$ ($[M+H]^+$) requires 221.9760.

2-Chloro-*N*-(2-oxotetrahydrofuran-3-yl)acetamide



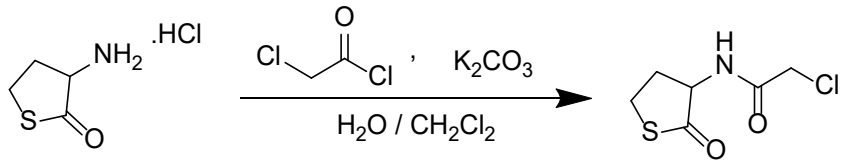
Method A: Chloroacetyl chloride (310 mg, 2.75 mmol, 0.22 mL), **36** (500 mg, 2.75 mmol), triethylamine (556 mg, 5.49 mmol). The reaction mixture was evaporated, and the crude product was purified via column chromatography (50% EtOAc in chloroform) to give **46** (334 mg, 1.88 mmol) in 68% yield as a white solid. $\mathbf{d_H}$ 7.05 (1H, br s, NH), 4.58 (1H, ddd, *J* 6.6, 8.6, 11.4 Hz, CH), 4.50 (1H, br t, *J* 9.0 Hz, CH), 4.31 (1H, ddd, *J* 5.9, 9.7, 10.8 Hz, CH), 4.10 (2H, s, CH_2), 2.81-2.88 (1H, m, CH), 2.17-2.28 (1H, m, 1H); $\mathbf{d_C}$174.6, 166.8, 66.1, 49.6, 42.3, 30.1; $\boldsymbol{\nu_{max}}$ 3251, 3069, 1762, 1662, 1556, 1179, 1024; **MS(CI)** 178.0 (100%, $[C_6H_8{}^{35}ClNO_3+H]^+$) 180.0 (30%, $[C_6H_8{}^{37}ClNO_3+H]^+$), 200.0 (100%, $[C_6H_8{}^{35}ClNO_3+Na]^+$), 202.0 (30%, $[C_6H_8{}^{37}ClNO_3+Na]^+$); HRMS(ES) found 178.0264, $C_6H_9{}^{37}ClNO_3{}^+$ ($[M+H]^+$) requires 178.0265.

2-Chloro-*N*-(2-oxotetrahydrofuran-3-yl)propanamide



Method A: Chloropropionyl chloride (349 mg, 2.75 mmol, 0.27 mL), **36** (500 mg, 2.75 mmol), trimethylamine (556 mg, 5.49 mmol). The reaction mixture was evaporated, and the crude product was purified via column chromatography (50% EtOAc in chloroform) to give **47** (341 mg, 1.78 mmol) in 65% yield as a white solid. $\mathbf{d_H}$ 6.99 (1H, br s, NH), 4.36-4.52 (3H, m, 3 x CH), 4.21-4.27 (1H, m, CH), 2.74-2.81 (1H, m, CH), 2.09-2.20 (1H, m, CH), 1.70/1.69 (3H, 2 x d, *J* 6.8/6.7 Hz, Me); $\mathbf{d_C}$ 174.7/174.7, 170.4/170.4, 66.1/66.1, 55.3/55.2, 49.6, 30.1/30.1, 22.5; $\boldsymbol{\nu_{max}}$ 3285, 3085, 1775, 1660, 1551, 1167; MS(CI) 192.0 (100%, $[C_7H_{10}{}^{35}ClNO_3+H]^+$) 194.0 (35%, $[C_7H_{10}{}^{37}ClNO_3+H]^+$), 214.0 (85%, $[C_7H_{10}{}^{35}ClNO_3+Na]^+$), 216.0 (25%, $[C_7H_{10}{}^{37}ClNO_3+Na]^+$); HRMS(ES) found 192.0422, $C_7H_{11}{}^{79}ClNO_3{}^+$ ($[M+H]^+$) requires 192.0422.

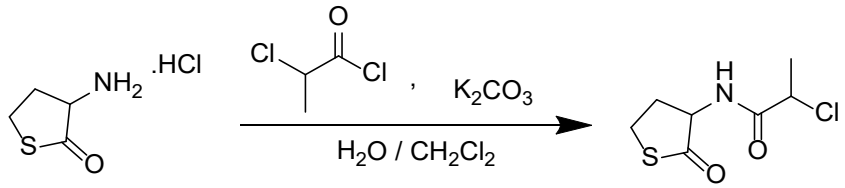
2-Chloro-*N*-(2-oxotetrahydrothiophen-3-yl)acetamide



37 **48**

Method B: Chloroacetyl chloride (753 mg, 6.51 mmol, 0.52 mL), **37** (500 mg, 3.25 mmol). The crude product was purified via column chromatography (50% EtOAc in chloroform) to give **48** (382 mg, 1.97 mmol) in 61% yield as a white solid. $\mathbf{d_H}$ 6.95 (1H, br s, NH), 4.52 (1H, apparent pentet, 6.5 Hz, CH), 4.12 (1H, d, *J* 15.1 Hz, CH), 4.07 (1H, d, *J* 15.1 Hz, CH), 3.39 (1H, ddd, *J* 5.1, 11.6, 11.6 Hz, CH), 3.29 (1H, br dd, *J* 7.0, 11.2 Hz, 1H), 2.91-2.98 (1H, m, CH), 2.00 (1H, dddd, *J* 7.0, 12.5, 12.5, 12.5 Hz, 1H); $\mathbf{d_C}$ 204.5, 166.7, 59.7, 42.5, 31.7, 27.7; $\boldsymbol{\nu_{max}}$ 3293, 2941, 1702, 1643, 1534, 1262; MS(CI) 194.0 (100%, $[C_6H_8{}^{35}ClNO_2S+H]^+$) 196.0 (35%, $[C_6H_8{}^{37}ClNO_2S +H]^+$), 216.0 (65%, $[C_6H_8{}^{35}ClNO_2S +Na]^+$), 218.0 (23%, $[C_6H_8{}^{37}ClNO_2S +Na]^+$); HRMS(ES) found 194.0037, $C_6H_9{}^{35}ClNO_2S^+$ ($[M+H]^+$) requires 194.0037.

2-Chloro-*N*-(2-oxotetrahydrothiophen-3-yl)propanamide



37 **49**

Method B: Chloropropionyl Chloride (826 mg, 6.51 mmol, 0.62 mL), **37** (500 mg, 3.25 mmol). The crude product was purified via column chromatography (50% EtOAc in chloroform) to give **49** (389 mg, 1.87 mmol) in 58% yield as a white solid. δ_H 6.93 (1H, s, NH), 4.41-4.52 (1H, m, 2 x CH), 3.38 (1H, td, *J*= 5.1, 11.8 Hz, CH), 3.28 (1H, dd, *J*= 7.0, 11.3 Hz, CH), 2.94 (1H, ddd, *J*= 0.9, 6.0, 12.1 Hz, CH), 1.99 (1H, ddd, *J*= 7.1, 12.4, 22.2 Hz, CH), 1.76/1.74 (2 x ½H, t, *J*= 7.1 Hz, CH_3); δ_C 212.8/212.8, 170.1/170.2, 59.6/59.7, 55.3/55.5, 31.5/31.5, 27.5/27.5, 22.5/22.6; $\boldsymbol{V_{max}}$ 3230, 3072, 1694, 1676, 1558, 1224; MS(CI) 208.0 (100%, $[C_7H_{10}{}^{35}ClNO_2S+H]^+$) 210.0 (35%, $[C_7H_{10}{}^{37}ClNO_2S +H]^+$), 230.0 (50%, $[C_7H_{10}{}^{35}ClNO_2S +Na]^+$), 232.0 (18%, $[C_7H_{10}{}^{37}ClNO_2S +Na]^+$); HRMS(ES) found 208.0193, $C_7H_{11}{}^{35}ClNO_2S^+$ ($[M+H]^+$) requires 208.0194.

References

1 P. A. Seabrook, MSc Thesis, Bangor University, 2015.

2 J. Tibble, MSc Thesis, Bangor University, 2016.

3 M. O. Ogbu, MSc Thesis, Bangor University, 2016.

4 C. Bernitzky, ERASMUS Project, Bangor University, 2016.

5 T. Feil, MSc Thesis, Bangor Univesity, 2015.

6 C. S. Stewart, H. J. Flint and M. P. Bryant, in *The Rumen Microbial Ecosystem*, Springer Netherlands, Dordrecht, 1997, pp. 10–72.

7 J. A. Frankel, *Greenhouse Gas Emissions*, 1999.

8 G. Flato, J. Marotzke, B. Abiodun, P. Braconnot, S. C. Chou, W. J. Collins, P. Cox, F. Driouech, S. Emori, V. Eyring, C. Forest, P. Gleckler, E. Guilyardi, C. Jakob, V. Kattsov, C. Reason and M. Rummukaines, in *Evaluation of Climate Models*, Cambridge University Press, 2013.

9 T. Andrews, J. M. Gregory, M. J. Webb and K. E. Taylor, *Geophys. Res. Lett.*, 2012, **39**, 1–7.

10 G. Zhan, X. Qu, J. Liu, Q. Tong, J. Zhou, B. Sun and G. Yao, *Sci. Rep.*, 2016, **6**, 33990.

11 A. D. C. Parenty, K. M. Guthrie, Y.-F. Song, L. V. Smith, E. Burkholder and L. Cronin, *Chem. Commun.*, 2006, 1194.

12 Z. Jin, *Nat. Prod. Rep.*, 2005, **22**, 111–126.

13 M. G. Banwell, D. W. Lupton, X. Ma, J. Renner and M. O. Sydnes, *Org. Lett.*, 2004, **6**, 2741–2744.

14 W. L. Chen, C. Y. Chen, Y. F. Chen and J. C. Hsieh, *Org. Lett.*, 2015, **17**, 1613–1616.

15 F. L. Warren and W. G. Wright, *J. Chem. Soc.*, 1958, 4696–4700.

16 P. C. M. van Zijl, B. H. Ruessink, J. Bulthuis and C. MacLean, *Acc. Chem. Res.*, 1984, **17**, 172–180.

17 P. Harper, PhD Thesis, Bangor University, 2016.

18 B. Gryseels, K. Polman, J. Clerinx and L. Kestens, *Lancet*, 2006, **368**, 1106–1118.

19 D. Engels, L. Chitsulo, A. Montresor and L. Savioli, *Acta Trop.*, 2002, **82**, 139–146.

20 R. Horton, *Lancet*, 2012, 380, 2053–2054.

21 R. Lozano and M. Naghavi, *Lancet*, 2012, **380**, 2095–2128.

22 R. D. Pearson and R. L. Guerrant, *Ann. Intern. Med.*, 1983, **99**, 195.

23 D. T. Felson, J. J. Anderson and R. F. Meenan, *Arthritis Rheum.*, 1990, **33**, 1449–1461.

24 P. J. Murphy, H. Whiteland and K. Hoffman, *Pers. Comm.*, 2015.

25 C. Fuqua, M. R. Parsek and E. P. Greenberg, *Annu. Rev. Genet.*, 2001, **35**, 439–468.

26 S.-Y. Kang, J. K. Lee, J.-H. Jang, B. Y. Hwang and Y.-S. Hong, *Microb. Cell Fact.*, 2015, **14**, 191.

27 D. G. Colley and W. E. Secor, *Parasite Immunol.*, 2014, **36**, 347–357.

Appendix – Chapter 1

Publication

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Slight changes in the chemical structure of haemanthamine greatly influence the effect of the derivatives on rumen fermentation *in vitro*

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Although the potential of plants extracts to improve feed efficiency and animal productivity and decrease methane emissions by enteric fermentation has been shown, the information available is often contradictory which has been attributed to differences in the complex mixture of bioactive compounds and their interactions. Understanding the degree to which structural features in a compound may affect the biological activity of an extract is essential. We hypothesised that relative small variations in the structure of a compound can have a significant influence on the ability of the derivatives to alter fermentation in the rumen. Nine compounds were synthetized from the natural alkaloid haemanthamine and tested *in vitro* for their effects on rumen protozoa and fermentation parameters. Our results showed that simple esterifications of haemanthamine or its derivative dihydrohaemanthamine with acetate, butyrate, pivalate or hexanoate led to compounds that differed in their effects on rumen fermentation.

Since the ban of antibiotics as growth promoting feed additives by the European Union in 2006, plant extracts and plant secondary metabolites (e.g. saponins, tannins and essential oils) have been widely investigated as alternatives to manipulate rumen fermentation[1]. Although the potential of plants extracts to increase productivity and decrease methane emissions has been shown[2], the information available is often contradictory with apparently similar products having different biological effects[3] (ie. saponin extracts/compounds differing in their ability to modulate fermentation *in vitro*[3,4]). This has been attributed to differences in the complex mixture of bioactive compounds and their interactions[5]. The composition of an extract can greatly vary according to the nature of the starting plant material (plant variety, harvest time, soil composition, altitude, climate, and processing and storage conditions) and the extraction and purification processes applied[3,6]. Indeed, large differences in composition between batches have been reported with saponin extracts even when prepared from the same substrate with the same methodology[3]. While standardized methods to ensure the homogeneity of plant extracts are needed, understanding the degree to which structural features in a compound may affect the biological activity of an extract is essential. Our hypothesis is that relative small variations in the structure of a compound can have a significant influence on the ability of such compounds to alter fermentation in the rumen.

Few studies on the structure-activity relationship that underly the mechanisms of action of pure compounds in the rumen have been published. Early studies by Bush *et al.*[7] with derivatives of perloline, one of the major alkaloids in tall fescue, showed that the effect on rumen fermentation was greatly influenced by the substitution at the C-5 position. We have recently revisited this concept, showing that modifications in the structure of Hederoside B, the major saponin present in ivy fruit extract, resulted in saponin-like analogues with different biological activities in terms of antiprotozoal effect and stability of the molecules in the rumen[4]. In the present work we attempt to expand the concept that relative small variations in the structure of a compound can have a

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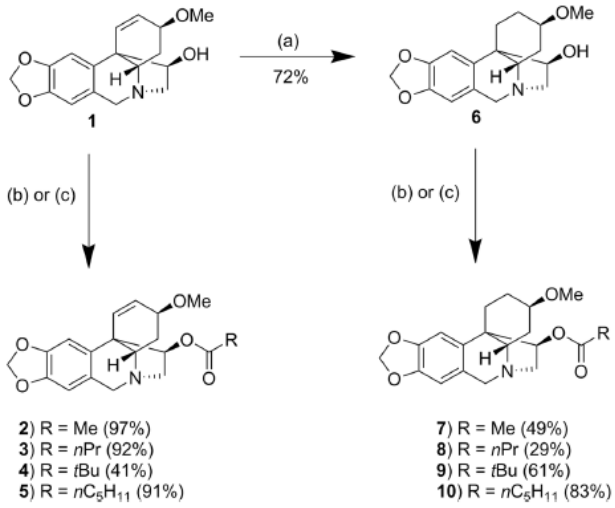


Figure 1. Production of derivatives from haemanthamine. (**a**) Under a nitrogen atmosphere, palladium on charcoal was added to a solution of haemanthamine **1** dissolved in dry tetrahydrofuran (THF). After evacuation, the mixture was stirred at room temperature under a hydrogen atmosphere for 16 hrs following which the reaction was filtered through a pad of Celite© which was washed with excess THF and the filtrate evaporated under reduced pressure. The residue was purified by flash column chromatography (to give dihydrohaemanthamine **6**). (**b**) Pyridine and DMAP were added to a solution of haemanthamine 1 or dihydrohaemanthamine **6** dissolved in dichloromethane. The mixture was cooled (0 °C) and acetic anhydride or the requried acid chloride was added slowly. The resultant mixture was stirred until complete consumption of starting material. The reaction was washed with NaOH solution and brine, dried and evaporated under reduced pressure. The crude product was purified using flash column chromatography. (**c**) Triethylamine and DMAP were added to a solution of haemanthamine **1** in dichloromethane. The mixture was cooled (0 °C) and the required acid chloride was added slowly and allowed to stir until complete consumption of the starting material. The sample was then washed subsequently with NaOH solution and brine then dried and evaporated under reduced pressure. The product was then purified using flash column chromatography.

significant influence on their biological activity by using the natural alkaloid haemanthamine to study the effect of simple esterifications of this molecule and its derivative dihydrohaemanthamine on both rumen protozoa and rumen fermentation pattern.

Results

Haemanthamine derivatives. Haemanthamine **1** was extracted from a fermented bulk of daffodil plants (*Narcissus Carlton*), from which galanthamine had been previously removed, and purified by recrystallization from acetone. The purified haemanthamine **1** was then used to produce a series of analogous compounds as shown in Fig. 1. The first derivatives obtained were the four simple esters (**2**–**5**) of acetate **2**, butyrate **3**, pivalate **4** and hexanoate **5**. The second series of derivatives were prepared from dihydrohaemanthamine **6**, which was obtained by the hydrogenation of haemanthamine **1**. Esterification of **6** was achieved in a similar manner as before and gave esters of acetate **7**, butyrate **8**, pivalate **9** and hexanoate **10**. Full synthesis and purification details and yields for each step together with structures are shown in Supplementary Material and in Supplementary Fig. S1

Antiprotozoal activity. Bacterial degradation by protozoa increased linearly ($R^2 > 0.99$) over 5 h in the control incubations. For each compound, the rate of bacterial degradation (% h^{-1}), a proxy of protozoal activity, at 0.125, 0.25, 0.5 and 1 g/L as compared with the control is shown in Supplementary Table S1 The inhibition of protozoa activity (Table 1) was significantly different between compounds and doses ($P < 0.001$). When added at 1 g/L, all compounds inhibited protozoa activity by 64–84%. Greater differences in the antiprotozoal effect between compounds were observed at lower concentrations, with dihydrohaemanthamine derivatives, and particularly derivative **10**, having a stronger effect.

Effect on fermentation parameters. The synthetized compounds, at 0.5 and 1 g/L, were tested further over 24 h in *in vitro* incubations (Tables 2 and 3). Overall, fermentation pattern was significantly different between

	Dose (g/L)			
	0.125	0.25	0.5	1
Haemanthamine derivatives				
2	13.9[a]	24.9[a]	38.3[b]	63.5[c]
3	0.2[a]	12.2[a]	72.1[b]	78.8[b]
4	10.7[a]	47.9[b]	79.7[c]	78.8[c]
5	7.7[a]	11.1[a]	55.8[b]	68.5[b]
Dihydrohaemanthamine derivatives				
6	17.4[a]	26.1[a]	42.5[b]	74.0[c]
7	3.8[a]	6.1[a]	32.9[b]	66.2[c]
8	4.8[a]	33.3[b]	81.8[c]	81.8[c]
9	17.9[a]	69.1[b]	89.3[c]	83.9[c]
10	41.7[a]	79.0[b]	78.8[bc]	83.5[c]
	SED		P	
Treatment	3.258		<0.001	
Dose	2.171		<0.001	
Treatment x Dose	6.516		<0.001	

Table 1. Inhibition of protozoa activity (% in respect to the control, no addition) by dihydrohaemanthamine and derivatives of haemanthamine and dihydrohaemanthamine, added at 0.125, 0.25, 0.5 or 1 g/L. SED: Standard error of the difference. [a–c]Means with different superscript differ significantly by dose within treatment.

compounds and doses ($P < 0.001$). Only total gas produced after 24 h was unaffected by the treatments, although all derivatives had an impact on methane emissions ($P < 0.001$).

Whereas slight increases in pH ($P < 0.001$) were observed in the presence of the synthetized compounds, the concentration of total volatile fatty acids (VFA) decreased ($P < 0.001$) by 19 and 26% when they were added at 0.5 and 1 g/L, respectively. Dihydrohaemanthamine **6**, its derivatives (**7**–**10**) and haemathamine derivatives (**2**–**5**) caused shifts in the molar proportions of VFA towards lower acetate and higher propionate ($P < 0.001$), to different extents depending on the compound. The greatest effect was observed with the dihydrohaemanthamine derivatives, and particularly **8**, **9** and **10**. For most of the synthetized compounds, whilst molar proportions of butyrate increased ($P < 0.001$), those of branched chain volatile fatty acids (BCVFA) decreased ($P < 0.001$). All compounds caused decreases in ammonia concentration ($P < 0.001$), this effect being greater with dihydrohaemanthamine **6** and the derivatives of haemanthamine (**2**–**5**).

Discussion

Alkaloids of the amaryllidaceae family have been reported to have a wide range of biological activities[8]. Some of these alkaloids are of particular interest because of their potential use in the treatment of protozoal diseases such as leshmaniasis, trypanosomiases and malaria[9], whilst antibacterial and antifungal activities have also been described[10].

Several studies aimed at developing antimalarial drugs on the relationship between the structure of novel synthesised compounds and their biological activity have been published[11–13]. Cedrón *et al.*[14] observed that haemanthamine derivatives with a methoxy group at C-3 and the presence of a free hydroxyl group at C-11 were more effective against protozoa than other derivatives; this antiprotozoal effect was also associated with the presence of a double bond at C1-C2[14].

The aim of this work was to study the relationship between the chemical structure of certain compounds and their effects on rumen protozoa and fermentation pattern; thus, nine compounds were synthetized from the natural alkaloid haemanthamine. The chemical modifications were carried out on the hydroxyl group at C-11 and/or on the double bond presents at C1-C2 in ring D. The hydrogenation of the double bond at C1-C2 of haemanthamine resulted in the derivative dihydrohaemanthamine **6**. Haemanthamine (**2**–**5**) or dihydrohaemanthamine (**7**–**10**) derivatives were obtained by esterification of the hydroxyl group at C-11 with acetate (**2** and **7**), butyrate (**3** and **8**), pivalate (**4** and **9**) or hexanoate (**5** and **10**). ^{1}H NMR and ^{13}C NMR spectra of each compound are shown in Supplementary Information.

All the compounds tested showed antiprotozoal effect that differed between treatments and doses, with dihydrohaemanthamine derivatives being more effective in inhibiting protozoal activity. Since the simple esters made the derivatives more lipophilic than the non-esterified molecule, it may have allowed them to cross the cell membrane of protozoa increasing then their antiprotozoal activity. The esterification of the hydroxyl group of dihydrohaemanthamine with hexanoate (derivative **10**) increased the antiprotozoal effect dramatically. However, the same modification of the haemanthamine molecule (derivative **5**) reduced the antiprotozoal effect. This decrease in activity, particularly at the lowest doses tested, was also observed with haemanthamine derivatives obtained by esterification with butyrate and pivalate (**3** and **4**) as compared with those of dihydrohaemanthamine with the same modifications in the structure (**8** and **9**). However, when the substituent was acetate, dihydrohaemanthamine derivative **7** was less effective inhibiting protozoa than the corresponding haemanthamine derivative **2** or

	Total VFA (mM)			Acetate (%)			Propionate (%)			Butyrate (%)			BCVFA (%)		
	Dose (g/L)			Dose (g/L)			Dose (g/L)			Dose (g/L)			Dose (g/L)		
	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
Haemanthamine derivatives															
2	62.9^{a}	50.7^{b}	46.7^{b}	58.8^{a}	48.0^{b}	47.4^{b}	16.4^{a}	24.6^{b}	23.8^{b}	18.5^{a}	21.3^{a}	22.3^{a}	2.70^{a}	2.56^{a}	2.56^{a}
3	62.9^{a}	52.1^{ab}	49.0^{b}	58.8^{a}	47.3^{b}	42.9^{b}	16.4^{a}	23.3^{b}	25.0^{b}	18.5^{a}	22.8^{ab}	25.8^{b}	2.70^{a}	2.68^{a}	2.55^{a}
4	62.9^{a}	55.6^{a}	52.5^{a}	58.8^{a}	48.7^{b}	46.0^{b}	16.4^{a}	23.2^{b}	23.9^{b}	18.5^{a}	21.9^{ab}	23.6^{b}	2.70^{a}	2.49^{a}	2.41^{a}
5	62.9^{a}	51.7^{a}	53.3^{a}	58.8^{a}	48.3^{b}	46.6^{b}	16.4^{a}	23.3^{b}	23.6^{b}	18.5^{a}	21.2^{a}	22.2^{a}	2.70^{a}	2.79^{a}	2.52^{a}
Dihydrohaemanthamine derivatives															
6	62.9^{a}	57.7^{a}	52.6^{a}	58.8^{a}	56.4^{a}	55.5^{a}	16.4^{a}	19.8^{a}	20.2^{a}	18.5^{a}	17.8^{a}	18.3^{a}	2.70^{a}	2.46^{a}	2.48^{a}
7	62.9^{a}	51.1^{b}	49.5^{b}	58.8^{a}	49.0^{b}	47.5^{b}	16.4^{a}	25.0^{b}	24.5^{b}	18.5^{a}	19.7^{a}	21.8^{a}	2.70^{a}	2.64^{a}	2.45^{a}
8	62.9^{a}	51.4^{b}	46.9^{b}	58.8^{a}	45.5^{b}	36.8^{c}	16.4^{a}	24.0^{b}	26.9^{b}	18.5^{a}	24.0^{b}	29.2^{b}	2.70^{a}	2.58^{a}	2.05^{b}
9	62.9^{a}	51.3^{b}	55.6^{ab}	58.8^{a}	44.6^{b}	41.6^{b}	16.4^{a}	26.2^{b}	33.9^{c}	18.5^{a}	22.0^{a}	17.0^{a}	2.70^{a}	3.27^{b}	4.18^{c}
10	62.9^{a}	55.6^{a}	62.3^{a}	58.8^{a}	41.3^{b}	41.2^{b}	16.4^{a}	27.2^{b}	37.9^{c}	18.5^{a}	22.3^{a}	10.5^{b}	2.70^{a}	2.28^{ab}	2.05^{b}
		SED	P		SED	P		SED	P		SED	P		SED	P
Treatment		1.630	0.001		1.110	<0.001		0.572	<0.001		0.643	<0.001		0.061	<0.001
Dose		0.943	<0.001		0.638	<0.001		0.330	<0.001		0.371	<0.001		0.035	0.004
Treatment x Dose		2.830	0.023		1.910	<0.001		0.991	<0.001		1.110	<0.001		0.106	<0.001

Table 2. Effect of dihydrohaemanthamine and derivatives of haemanthamine and dihydrohaemanthamine (added at 0.5 and 1 g/L) on total (mM) and individual (% of total VFA) after 24 h of incubation. VFA: volatile fatty acids; BCVFA: branched chain volatile fatty acids; SED: Standard error of the difference. $^{a-b}$Means with different superscript differ significantly by dose within treatment.

	pH			Ammonia (mM)			Total gas (mL)			Methane (mL)		
	Dose (g/L)			Dose (g/L)			Dose (g/L)			Dose (g/L)		
	0	0.5	1	0	0.5	1	0	0.5	1	0	0.5	1
Haemanthamine derivatives												
2	6.19^{a}	6.23^{ab}	6.36^{b}	7.12^{a}	5.16^{b}	6.06^{ab}	25.1^{a}	21.36^{ab}	18.04^{b}	2.99^{a}	0.005^{b}	0.003^{b}
3	6.19^{a}	6.29^{ab}	6.38^{b}	7.12^{a}	6.51^{a}	6.34^{a}	25.1^{a}	22.09^{ab}	19.77^{b}	2.99^{a}	0.003^{b}	0.003^{b}
4	6.19^{a}	6.17^{a}	6.37^{b}	7.12^{a}	5.24^{b}	6.17^{ab}	25.1^{a}	23.56^{ab}	17.77^{b}	2.99^{a}	1.0^{b}	0.48^{b}
5	6.19^{a}	6.29^{a}	6.27^{a}	7.12^{a}	7.04^{a}	6.07^{a}	25.1^{a}	22.10^{ab}	21.59^{a}	2.99^{a}	0.003^{b}	0.003^{b}
Dihydrohaemanthamine derivatives												
6	6.19^{a}	6.25^{ab}	6.36^{b}	7.12^{a}	5.38^{b}	5.85^{ab}	25.1^{a}	21.95^{ab}	18.96^{b}	2.99^{a}	1.94^{a}	1.62^{a}
7	6.19^{a}	6.31^{ab}	6.34^{b}	7.12^{a}	5.72^{b}	6.12^{ab}	25.1^{a}	21.61^{ab}	19.61^{b}	2.99^{a}	0.003^{b}	0.003^{b}
8	6.19^{a}	6.33^{b}	6.37^{b}	7.12^{a}	6.65^{a}	6.76^{a}	25.1^{a}	21.04^{ab}	19.80^{b}	2.99^{a}	0.003^{b}	0.003^{b}
9	6.19^{a}	6.31^{ab}	6.33^{b}	7.12^{a}	6.94^{a}	6.71^{a}	25.1^{a}	20.29^{ab}	17.75^{b}	2.99^{a}	0.003^{b}	0.003^{b}
10	6.19^{a}	6.26^{a}	6.25^{a}	7.12^{a}	6.86^{a}	6.08^{a}	25.1^{a}	19.35^{b}	18.38^{b}	2.99^{a}	0.003^{b}	0.003^{b}
		SED	P		SED	P		SED	P		SED	P
Treatment		0.019	0.020		0.190	<0.001		0.730	0.176		0.251	<0.001
Dose		0.011	<0.001		0.110	<0.001		0.421	<0.001		0.145	<0.001
Treatment x Dose		0.033	<0.001		0.329	<0.001		1.26	0.330		0.434	0.154

Table 3. Effect of dihydrohaemanthamine and derivatives of haemanthamine and dihydrohaemanthamine, added at 0.5 and 1 g/L, on pH, ammonia (mM) and total gas and methane (mL) produced after 24 h of incubation. SED: Standard error of the difference. ^{a,b}Means with different superscript differ significantly by dose within treatment.

the dihydrohaemanthemine molecule. Our results indicate that the double bond present at C1-C2 in ring D may only play a role in the antiprotozoal activity when combined with certain chemical modifications in the structure.

Although the concentration of total VFA was reduced by the synthetized compounds, as compared with the control (no derivative added), a shift in the molar proportion of VFA towards propionate and, to a lesser extend butyrate, at expenses of acetate was observed across treatments. While the increase in propionate was substantial with **9** and **10** (33.9 and 37.9%, respectively, vs 16.4%), increases in butyrate were not observed in incubations with these compounds.

Dihydrohaemanthamine derivatives had the greatest effect on the molar proportions of VFA, which is in line with the effects observed on protozoa activity, followed by haemanthamine derivatives and

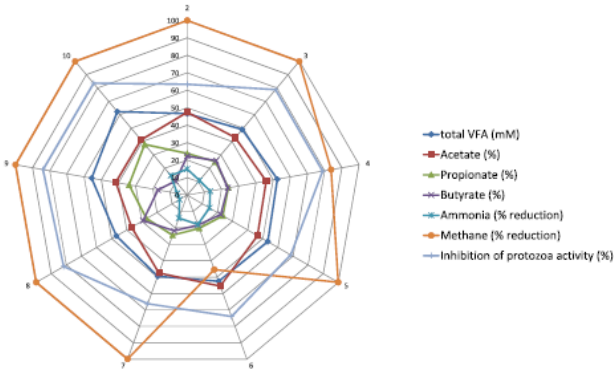


Figure 2. Effect of dihydrohaemanthamine **6** and derivatives of haemanthamine (**2–5**) and dihydrohaemanthamine (**7–10**), added at 1 g/L, on fermentation pattern after 24 h of incubation. Each axis represents one compound tested. Each point in the plot represents a value for total VFA (mM), molar proportions of acetate, propionate and butyrate, ammonia or methane reduction (percentage in respect to the control, no addition) and inhibition of protozoa (percentage in respect to the control, no addition).

dihydrohaemanthamine. Similar effects on molar proportions of VFA were observed when acetate was esterified to haemanthamine or dihydrohaemanthamine, contrary to what was observed when studying protozoal activity.

Interestingly, dihydrohaemanthamine and derivatives of haemanthamine were more effective in reducing ammonia concentration (by 18–24% and 9–27%, respectively) than dihydrohaemanthamine derivatives, being **8** and **9** the less effective compounds (2.5–6% reduction). The reduction in ammonia in the presence of the derivatives could be explained by the observed effect on protozoa, involved in the turnover of bacterial protein in the rumen[15], and possibly an inhibitory effect on high ammonia-producing bacteria. The esterification of the dihydrohaemanthamine molecule, however, decreased the ability of the compounds to reduce ammonia, as compared with that observed for dihydrohaemantamine.

All derivatives seemed to have a remarkable effect on methane emissions; whereas dihydrohaemanthemine **6** and compound **4** decreased methane by 35–45% and 66–84%, respectively, the rest of derivatives caused almost the complete inhibition of methane production, even at the lowest concentration tested. This may suggest a direct effect of the derivatives on the population of methanogenic archaea.

The different effects of the compounds added at 1 g/L on fermentation parameters are illustrated in Fig. 2 in which molar proportions of VFA, the percentage of reduction of ammonia and methane, and the percentage of inhibition of protozoa activity are plotted in a figure with 9 axes, one for each compound. The esterification of dihydrohaemanthamine with hexanoate, acetate, butyrate or pivalate led to a greater effect on VFA, although it did not seem to have the same impact on ammonia concentration, as compared with the effects observed with the rest of derivatives. The esterification of either haemanthamine or dihydrohaemanthamine did not seem to enhance the inhibitory effect on ammonia, as a greater reduction was observed with dihydrohaemanthamine. All the esterified compounds, however, were more effective in decreasing acetate and increasing propionate molar proportions than the dihydrohaemanthamine molecule. All derivatives, when added at 1 g/L, showed a great antiprotozoal effect and inhibited, almost completely, methane production.

It appears that whilst certain modifications in the chemical structure may influence some aspect of rumen fermentation, they may not necessary have an effect on others. Since the stability of the compounds overtime was not evaluated, it cannot be ruled out that the observed effects on fermentation could be also due to the potential breakdown products obtained from these derivatives. Studies to characterize the changes in rumen microbial communities associated with the effects of these derivatives on rumen fermentation are needed to understand which structural features may play a role in improved feed efficiency. The evaluation of the effects of other substituents and/or more than one variant in the structure as well as the synergistic effects is also suggested. However, it is clear that relative small variations in the structure of a compound can have a significant influence on their biological activity in terms of the ability to manipulate gut fermentation. Thus, future studies on the effect of plant extracts on fermentation in the gut of farm and companion animals need to combine detailed chemical characterisation of the extract used together with examination of the biological effects in the gut.

Methods

Synthesis of haemanthamine derivatives. Haemanthamine (extracted from Narcissus Carlton) was provided by Agroceutical Ltd. Synthesis pathways used are summarised in Fig. 1 and described in detail in the Supplementary Information.

The purity of the synthesised compounds was established by quantitative nuclear magnetic resonance (qNMR) spectroscopy using a Bruker Ultrashielded 400 spectrometer (Bruker Corporation, Coventry, UK) confirming purities of >98% for all derivatives.

Measurement of protozoal activity. The effect of dihydrohaemanthamine and derivatives of haemanthamine and dihydrohaemanthamine on protozoal activity was measured *in vitro* as the breakdown of [^{14}C]-labelled bacteria by rumen protozoa as described by Wallace and McPherson[16]. Isotope-labelled bacteria were obtained by growing *Streptococcus bovis* ES1 in Wallace and McPherson media[16] containing [^{14}C] leucine (1.89 µCi/7.5 mL tube) as the sole nitrogen source, for 24 h. Cultures were centrifuged (3,000 g, 15 min), supernatant discarded and pellets re-suspended in 7 mL of simplex type salt solution[17] (STS) containing ^{12}C-leucine (5 mM). This process was repeated three times to prevent re-incorporation of released [^{14}C] leucine by bacteria.

Rumen digesta was obtained from four rumen-cannulated Holstein-Frisian cows (four replicates) fed at maintenance level (composed of perennial ryegrass hay and concentrate at 67:33 on a DM basis). Animal procedures were carried out in accordance with the Animal Scientific Procedures Act 1986 and protocols were approved by the Aberystwyth University Ethical Committee. Rumen digesta was obtained before the morning feeding and strained through two layers of muslin and diluted with STS (1:1) containing ^{12}C-leucine (5 mM). Diluted rumen fluid (7.5 mL) was then incubated with labelled bacteria prepared as described above (0.5 mL) in tubes containing no additive (control) or 0.125, 0.25, 0.5 or 1 g/L of the compounds. Incubations were carried out at 39 °C under a stream of CO_2 and tubes were sampled at time 0 and at 1 h intervals up to 5 h using a syringe with a 19 gauge needle. Samples (0.5 mL) were acidified (by adding 0.125 mL of 25% trichloroacetic acid (wt/vol) and centrifuged (13,000 *g*, 5 min). Supernatant (0.200 mL), was diluted with 2 mL of OptiPhase HiSafe 2 scintillation fluid (Perkin Elmer, Seer Green, UK) to determine the radioactivity released by liquid-scintillation spectrometry (Hidex 300 SL, Lablogic Systems Ltd, Broomhill, UK). Bacterial breakdown at each incubation time was expressed as the percentage of the acid-soluble radioactivity released relative to the total radioactivity present in the initial labelled bacteria[16].

A simple linear regression was conducted to model the relationship between the percentage of radioactivity released (relative to the ^{14}C-bacterial inoculum) and the time (from 0 h to 5 h), as well as its correlation coefficient. The slope of this trend-line indicated the bacterial degradation rate (as % h^{-1}) and ultimately acts as a proxy of protozoal activity.

Determination of rumen fermentation pattern. To measure the short term effect of dihydrohaemanthamine and derivatives of haemanthamine and dihydrohaemanthamine on fermentation parameters, 24 h *in vitro* incubations were carried out. The experimental design consisted of a control (no additive) and the compounds added at 0.5 or 1 g/L. The experiment was conducted in quadruplicate, using rumen fluid from the same four cannulated cows. Rumen contents were sampled before the morning feeding, filtered through a double layer of muslin and diluted 1:2 in artificial saliva solution[18]. Aliquots (10 mL) of the diluted strained rumen fluid were added anaerobically to 40 mL Wheaton bottles containing 0.1 g of diet composed of ryegrass hay and barley (40:60), previously ground to pass through a 1-mm^2 mesh screen. Bottles were sealed and incubated at 39 °C receiving a gentle mix before sampling.

After 24 h of the incubation, gas was measured using a pressure transducer. After pressure was released, a gas sample (0. 5 mL) was collected from the headspace and immediately injected in a chromatograph (ATI Unicam 610 Series, Unicam Ltd., Cambridge, UK), fitted with a 40 cm Porapak N metal packed column (Agilent, Cheshire, UK) and flame ionization detector, to determine methane concentration. Then, bottles were opened, pH measured and a sample was collected and divided in two subsamples: one of the subsamples (4 mL) was diluted with 1 mL of deproteinising solution (200 mL/L orthophosphoric acid containing 20 mmol/L of 2-ethylbutyric acid as an internal standard) for the determination of VFA using gas chromatography, as described by Stewart and Duncan[19]. Another subsample (1 mL) was diluted with 0.250 mL of 25% trichloroacetic acid (wt/vol) for analysis of ammonia using a colorimetric method[20].

Statistical analyses. Trend line slopes were analysed statistically by randomized block ANOVA, with individual cows as a blocking term. Inhibition of protozoa activity (% with respect to the control) and fermentation parameters were analysed using ANOVA with treatment, dose and their interaction as fixed effects and cow as blocking term. When significant effects were detected across the different doses, means were compared by Fisher's unprotected LSD test.

Data Availability

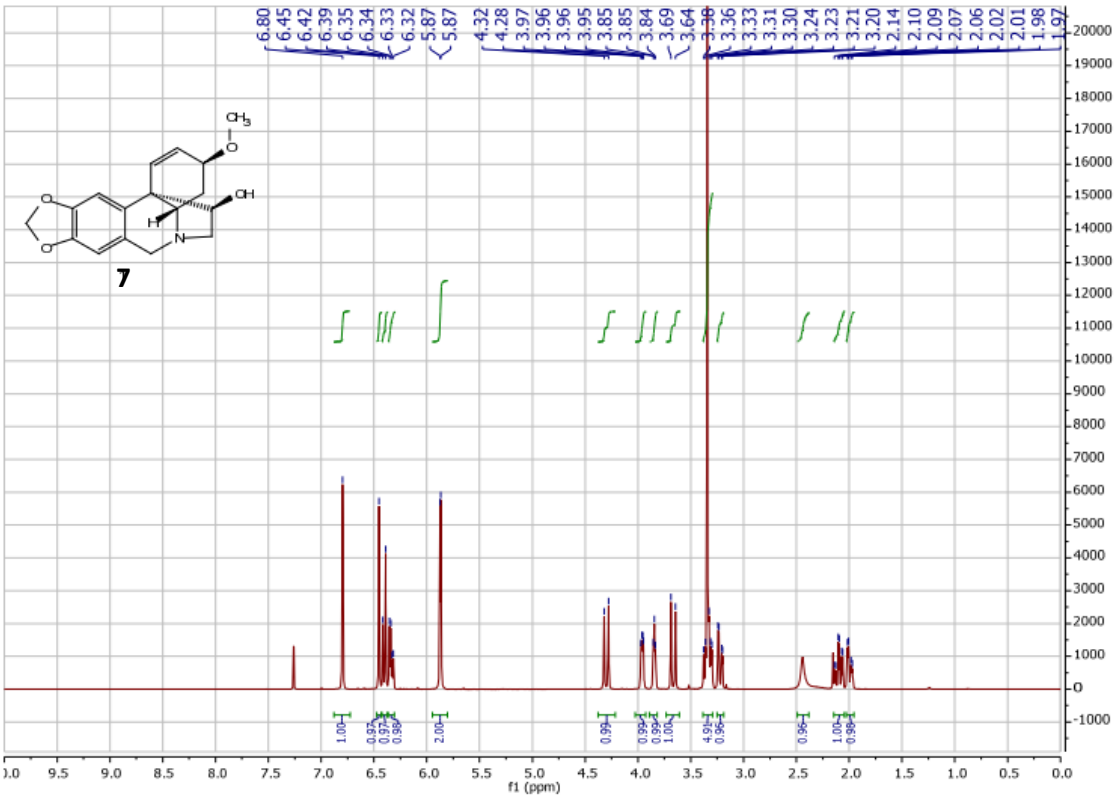
All data generated or analysed during this study are included in the published article (and its Supplementary Information files).

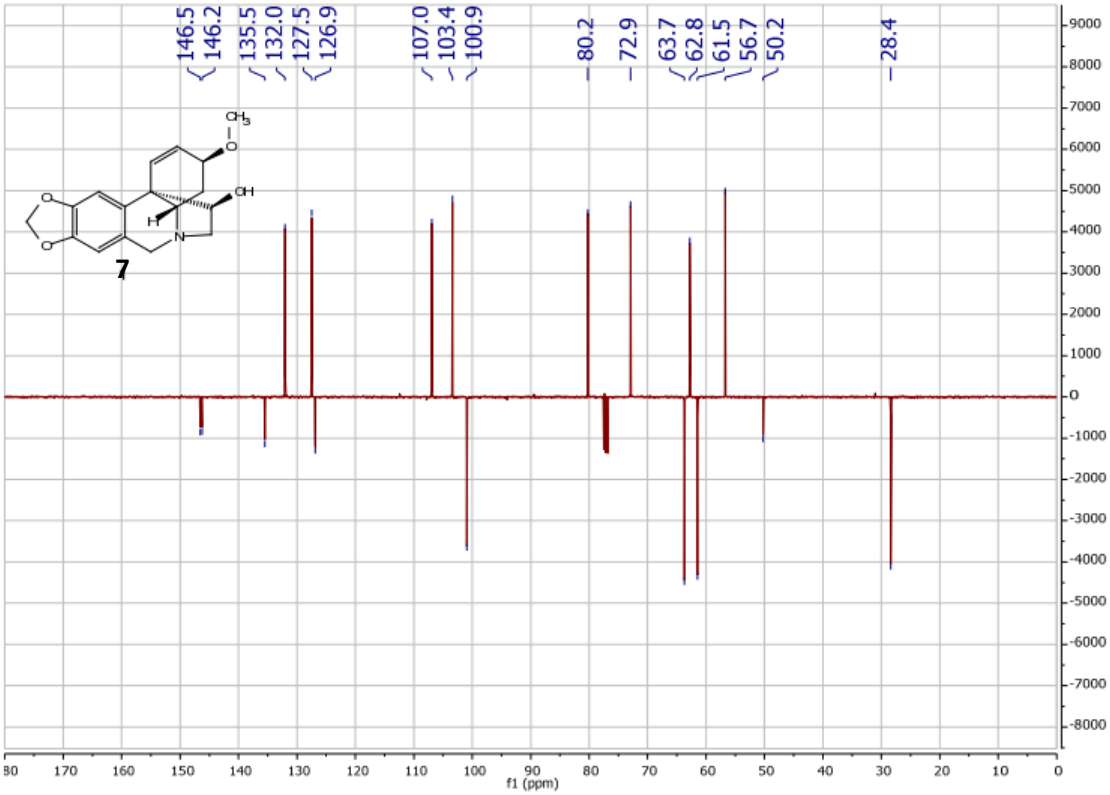
References

1. Hart, K. J., Yañez-Ruiz, D. R., Duval, S. M., McEwan, N. R. & Newbold, C. J. Plant extracts to manipulate rumen fermentation. *Anim. Feed Sci. Technol.* **147**, 8–35 (2008).
2. Bodas, R. *et al.* Manipulation of rumen fermentation and methane production with plant secondary metabolites. *Anim. Feed Sci. Tech.* **176**, 78–93 (2012).
3. Cieslak, A., Szumacher-Strabel, M., Stochmal, A. & Oleszek, W. Plant components with specific activities against rumen methanogens. *Animal* 7, 253–265 (2013).
4. Ramos-Morales, E. *et al.* Antiprotozoal effect of saponins in the rumen can be enhanced by chemical modifications in their structure. *Front. Microbiol.* **8**, 399, https://doi.org/10.3389/fmicb.2017.00399 (2017).
5. Atanasov, A. G. *et al.* Discovery and resupply of pharmacologically active plant-derived natural products: A review. *Biotechnol. Adv.* **33**, 1582–1614 (2015).
6. Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K. M. & Yoga Latha, L. Extraction, isolation and characterization of bioactive compounds from plants extracts. *Afr. J. Tradit. Complement. Altern. Med.* **8**, 1–10 (2011).
7. Bush, L. P., Burton, H. & Boling, J. A. Activity of tall fescue alkaloids and analogues in *in vitro* rumen fermentation. *J. Agric. Food Chem.* **24**, 869–872 (1976).
8. Ding, Y. *et al.* Phytochemical and biological investigations of Amaryllidaceae alkaloids: a review. *J. Asian Nat. Prod. Res.* **19**, 53–100 (2017).

NMR Spectra of compounds 7-16

7





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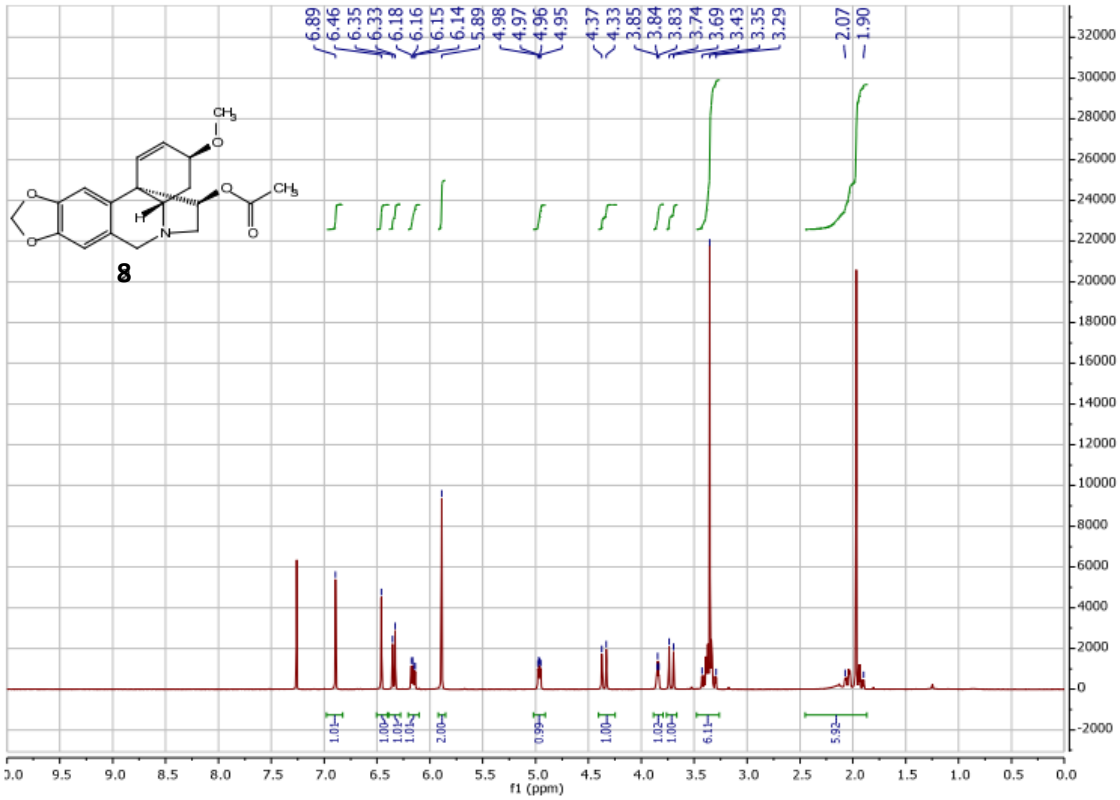
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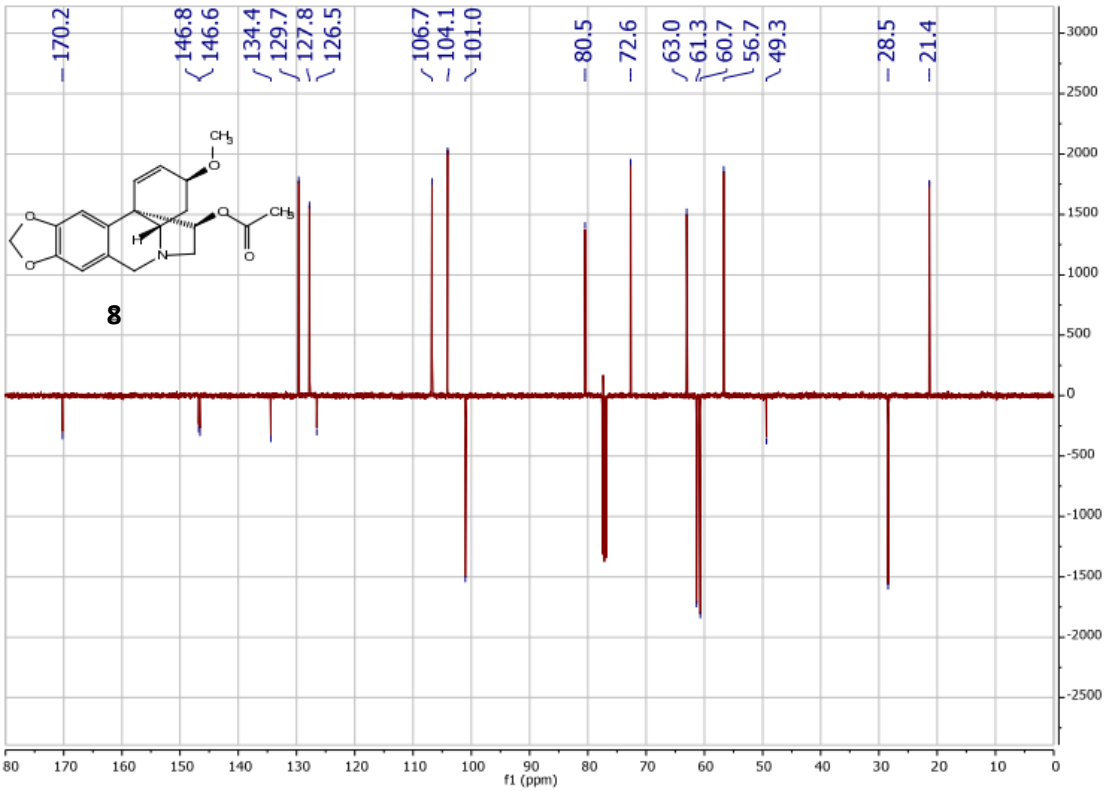
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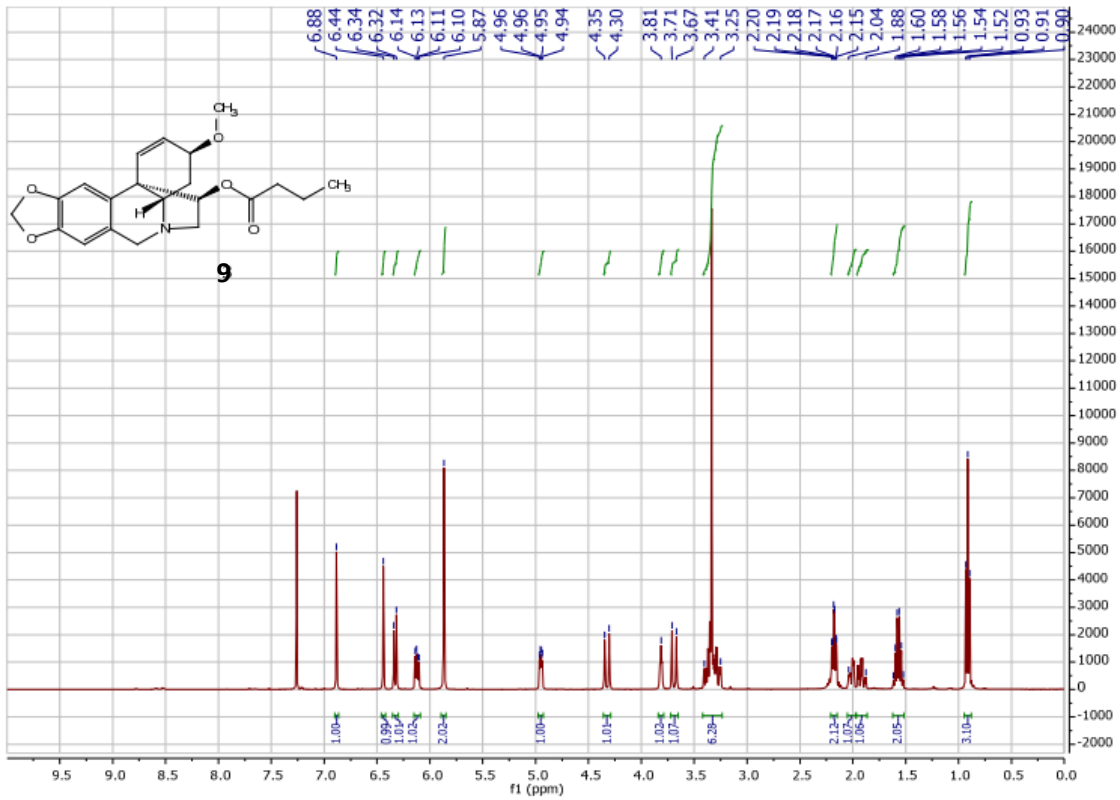
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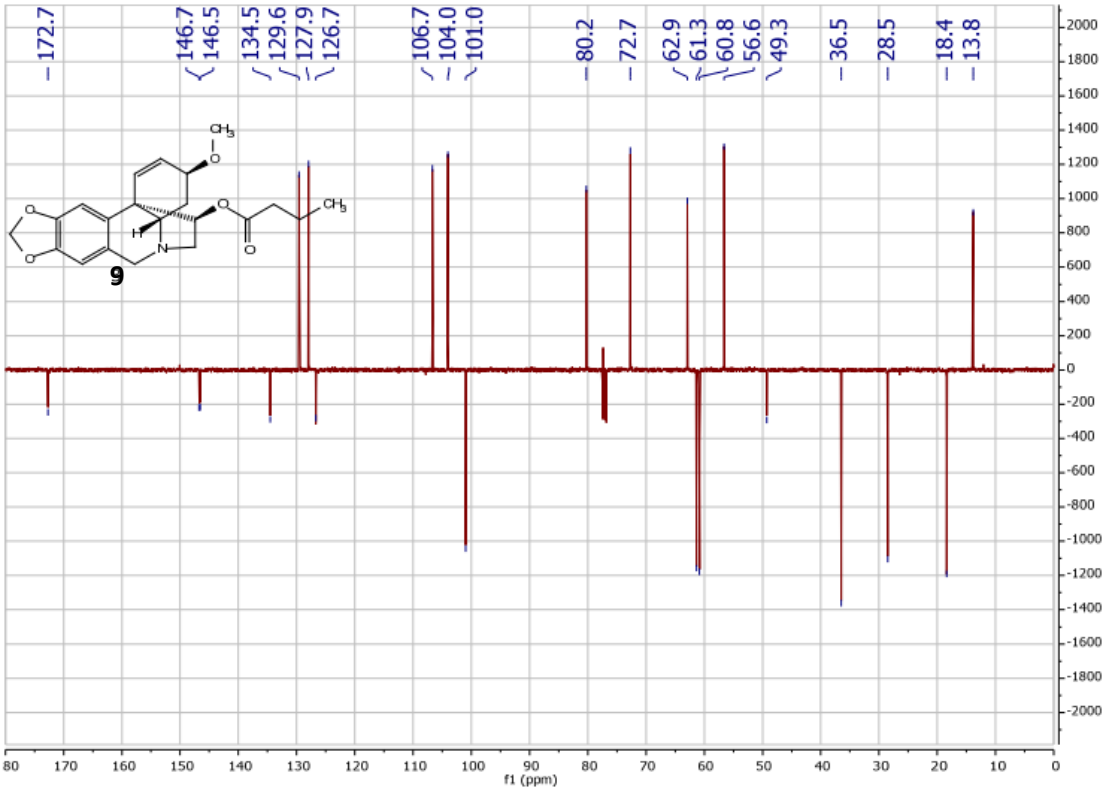
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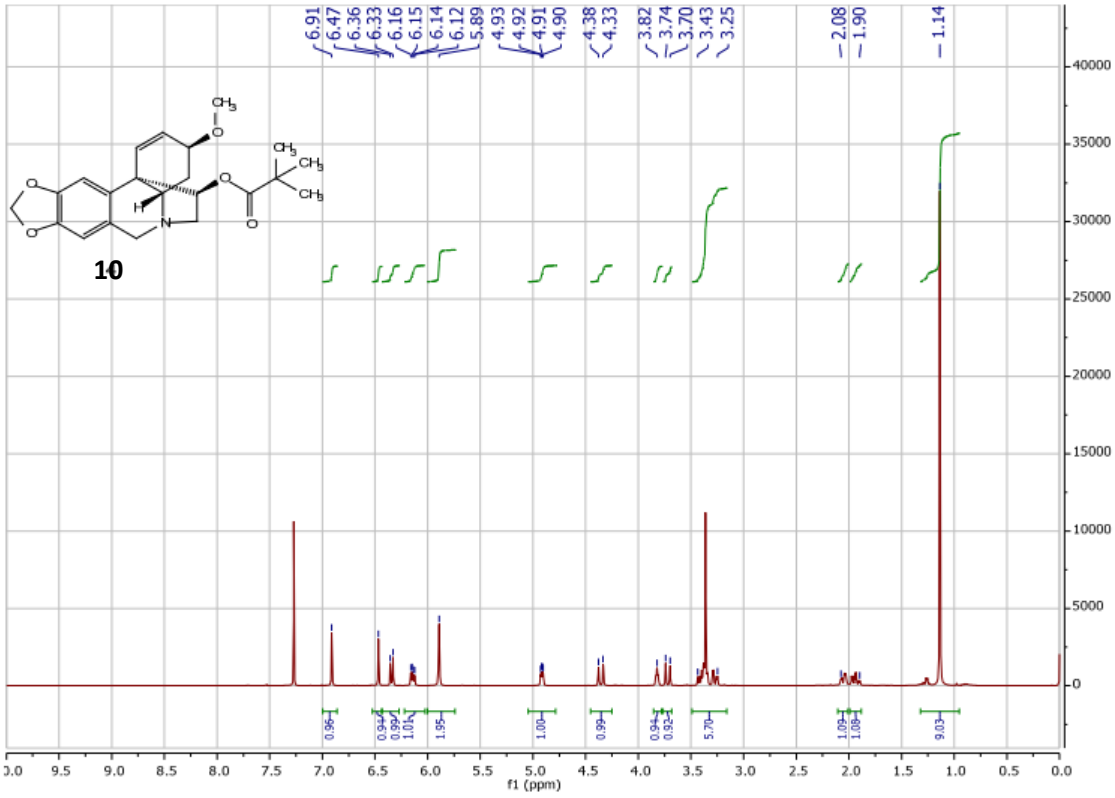
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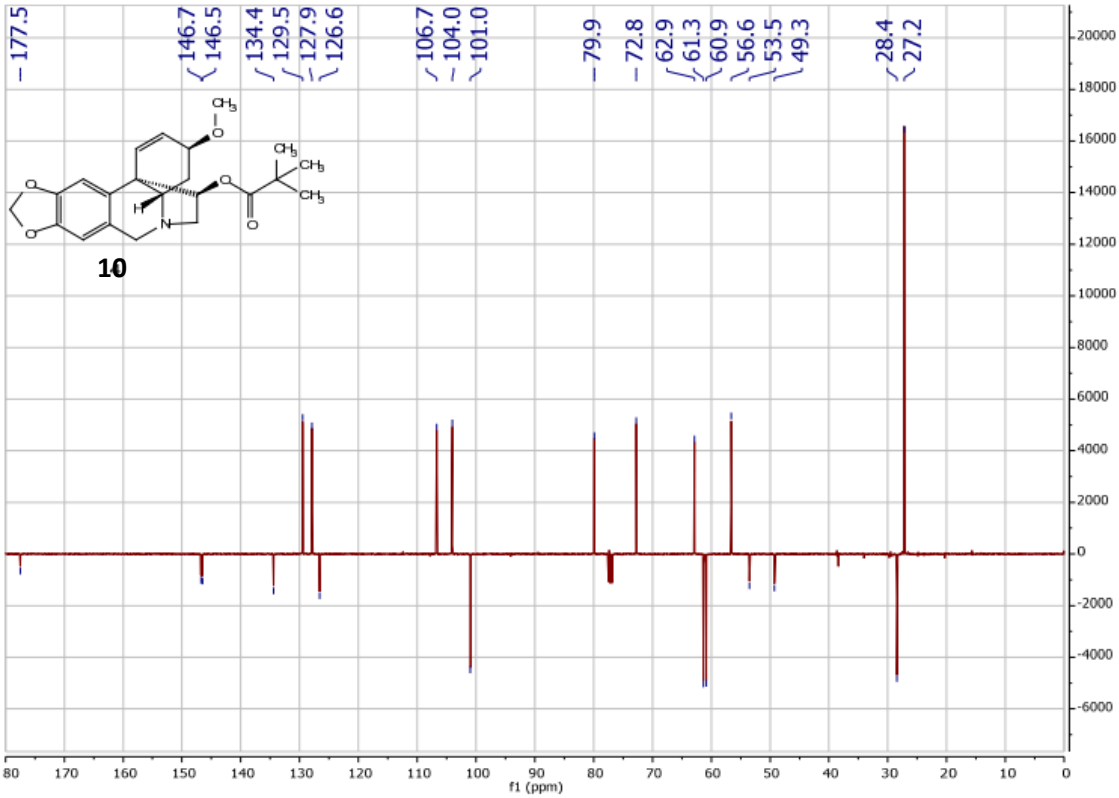
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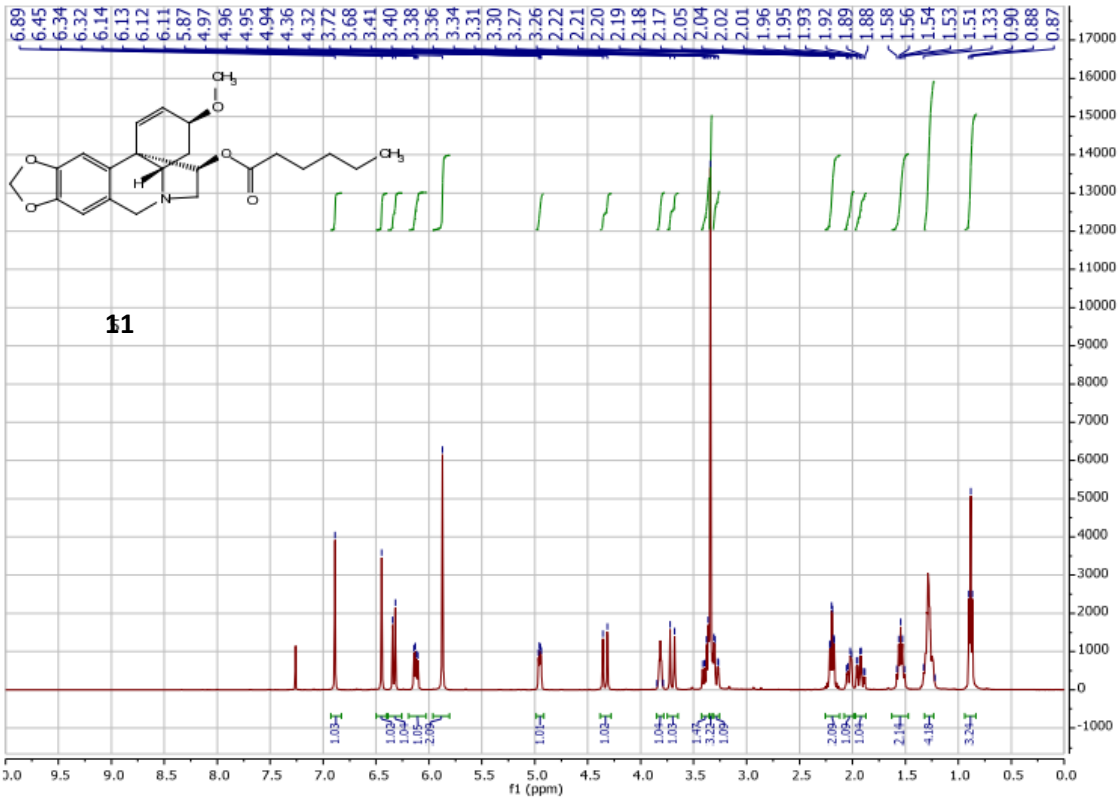

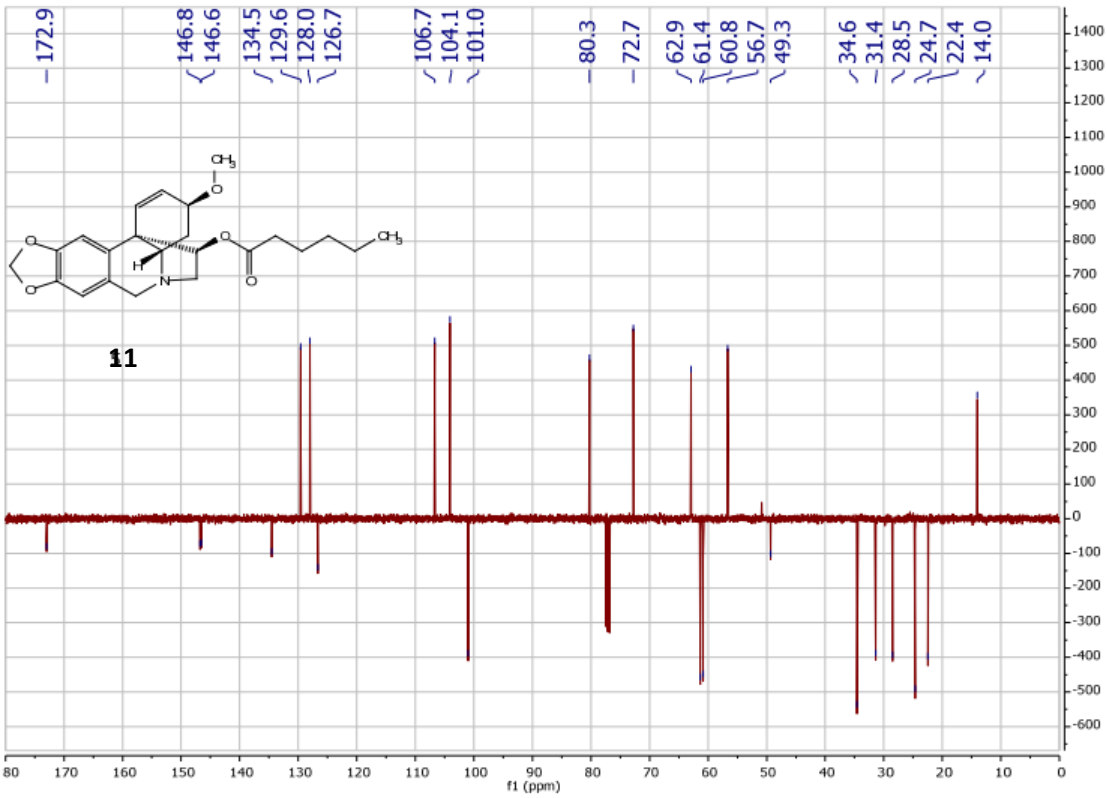

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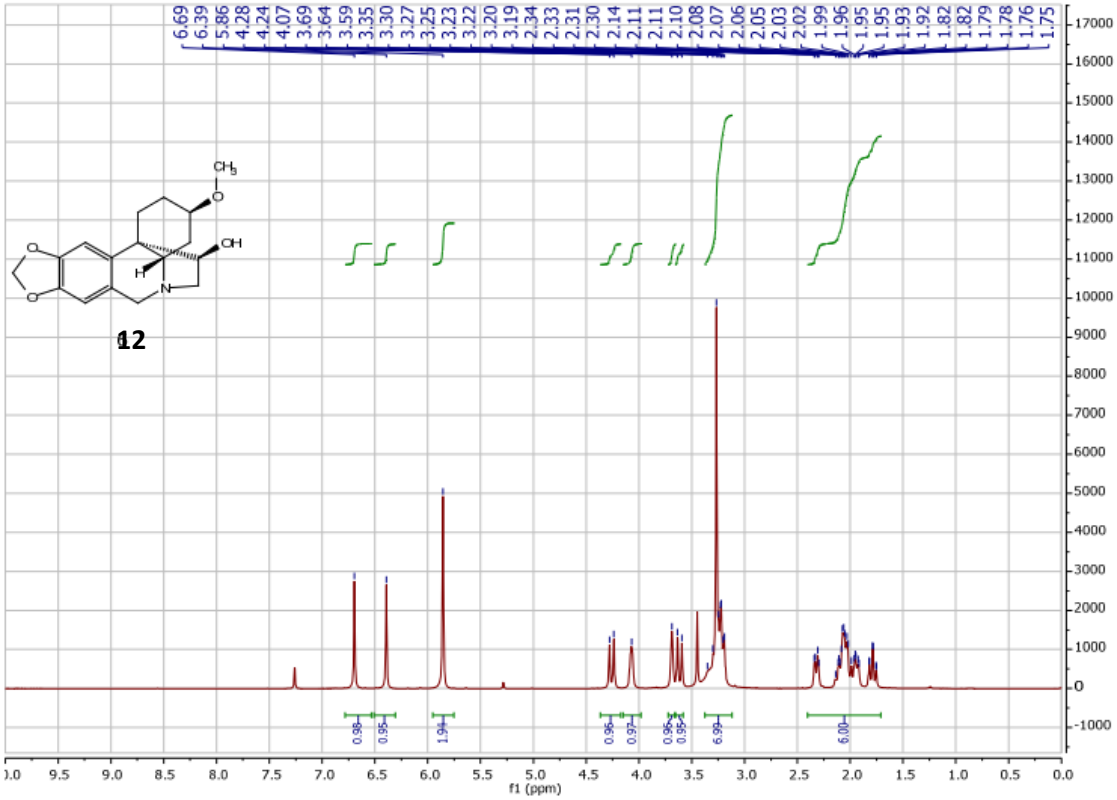
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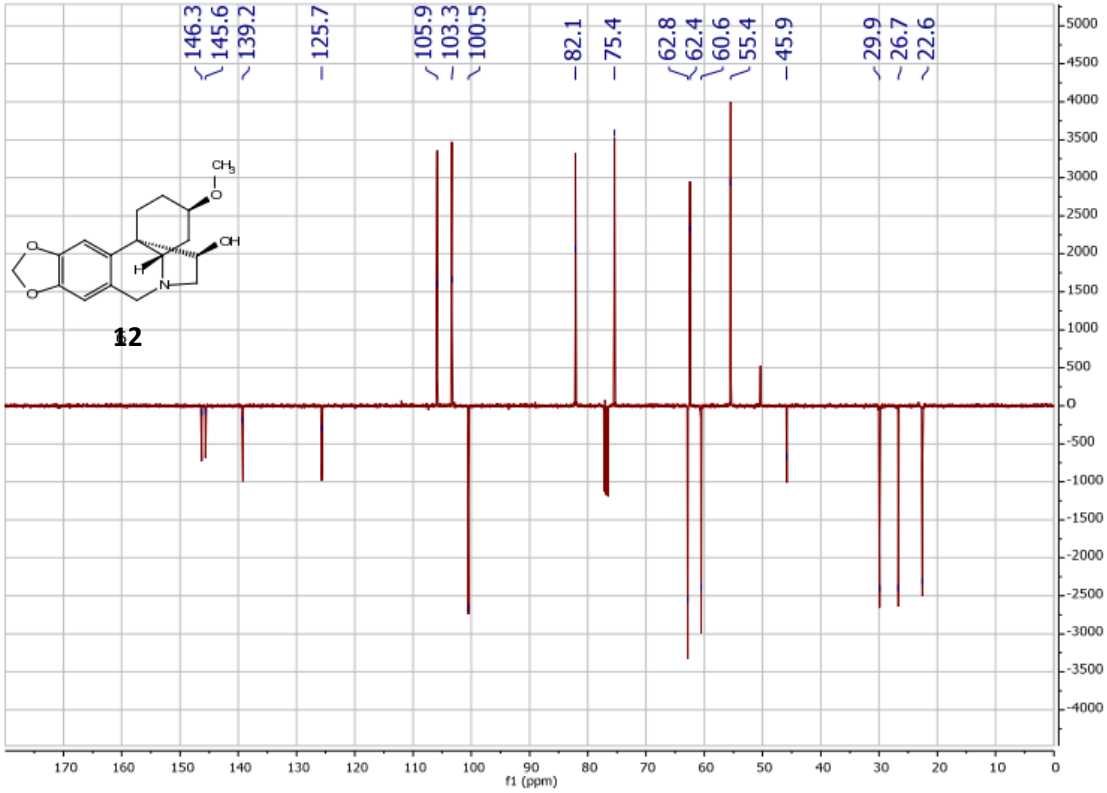
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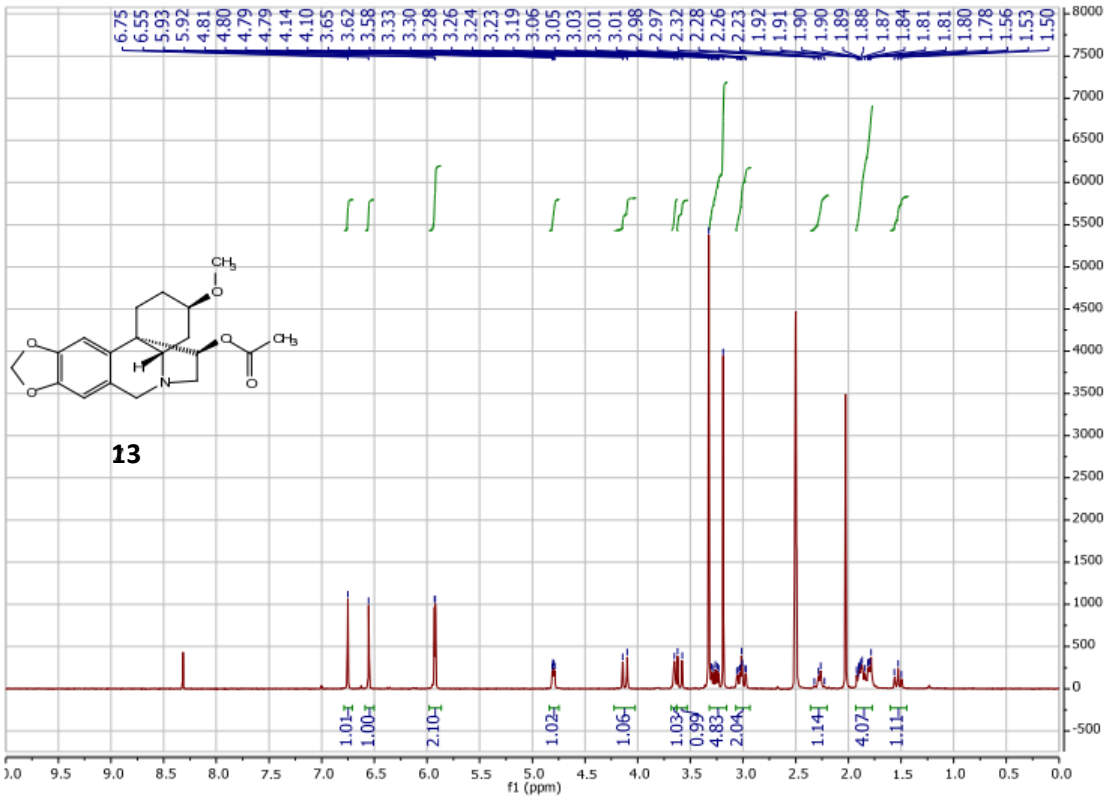
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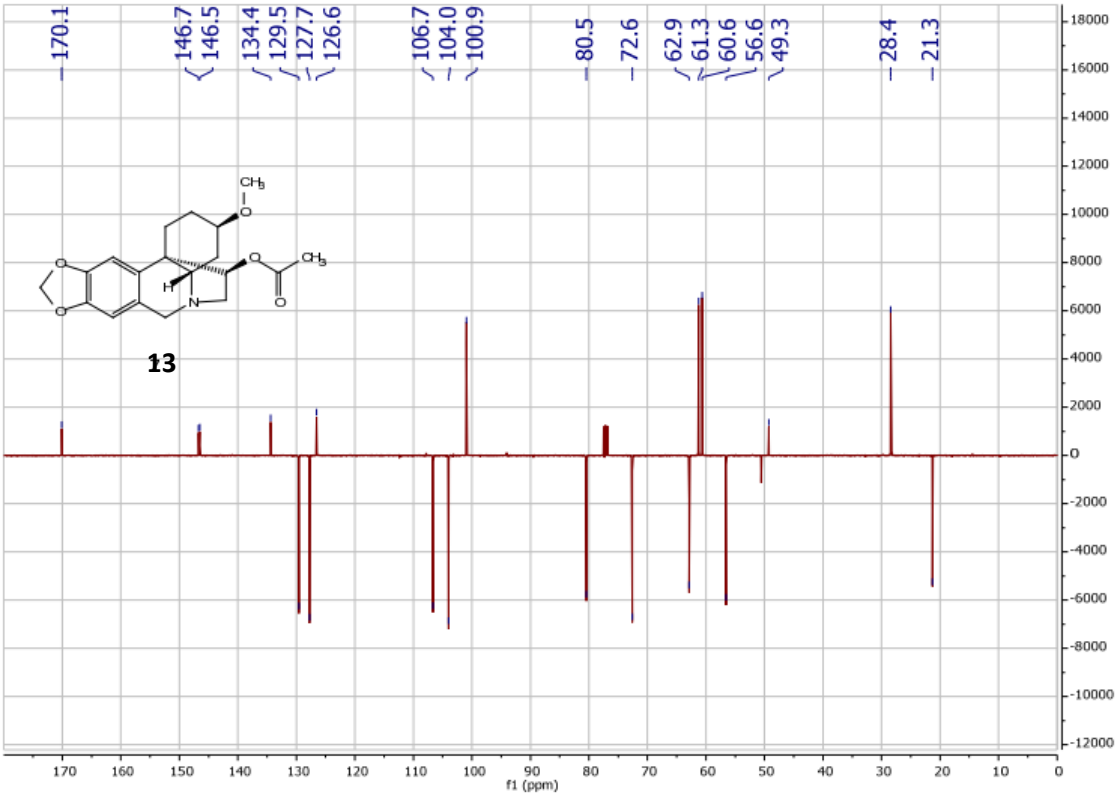
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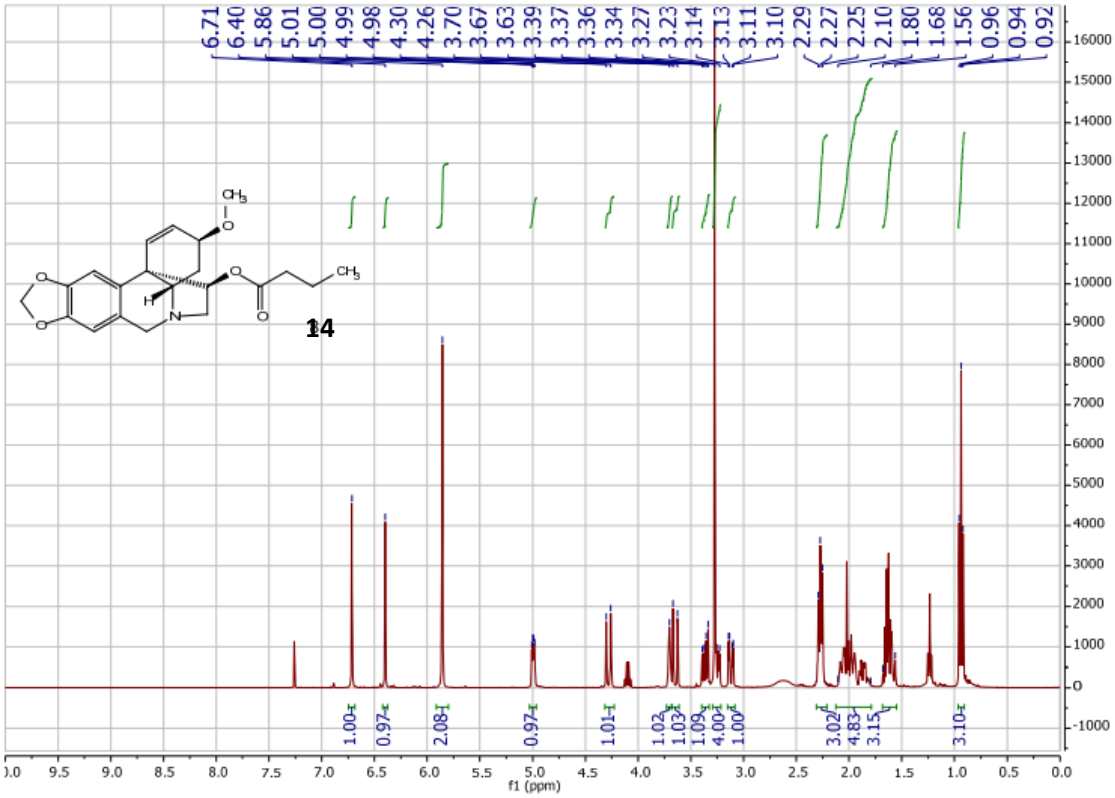
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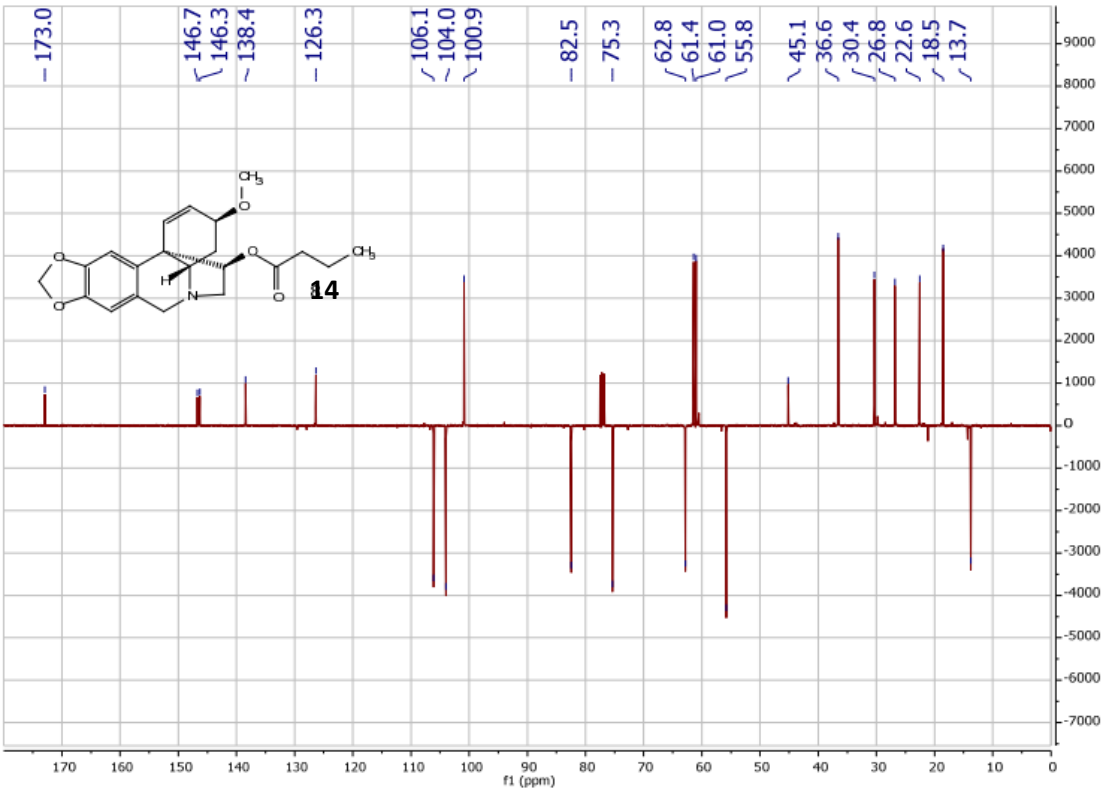
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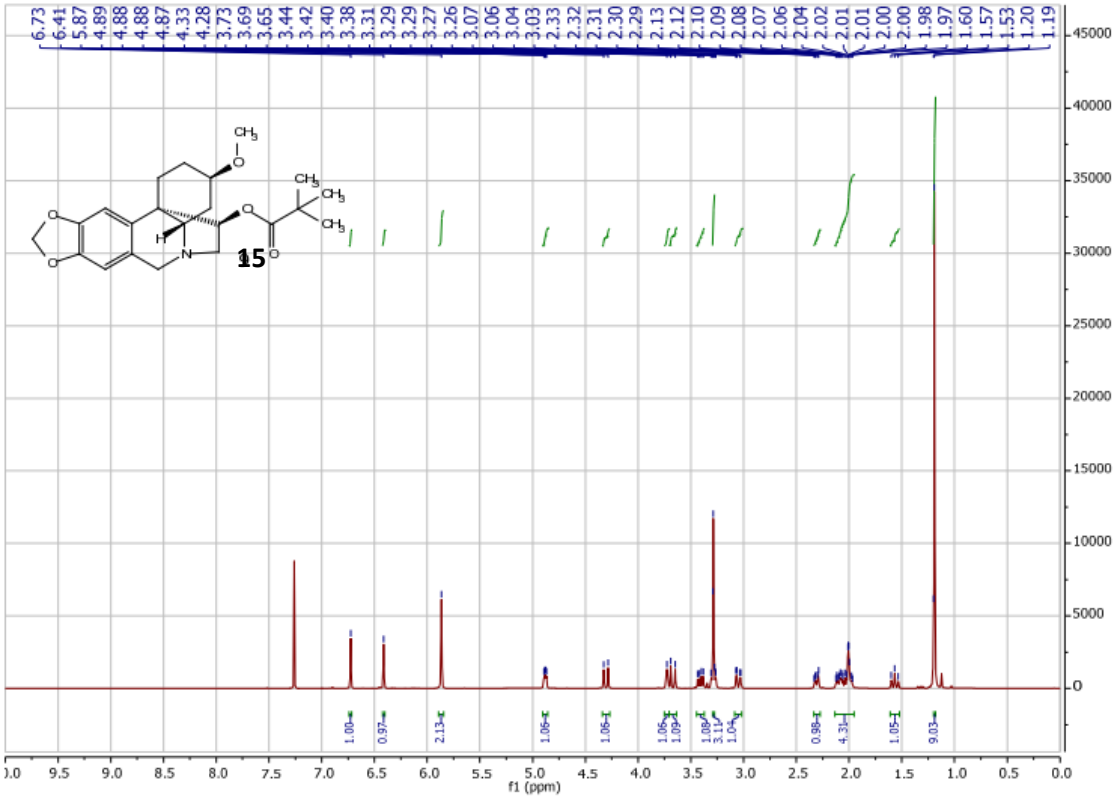
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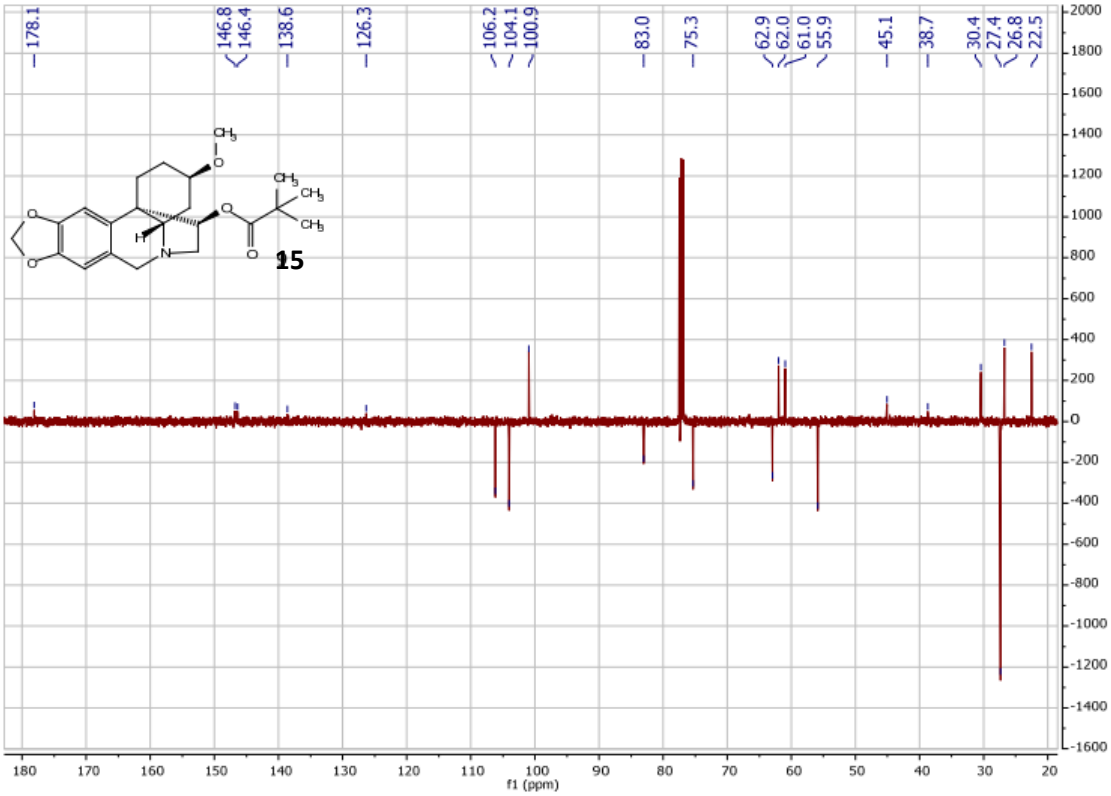
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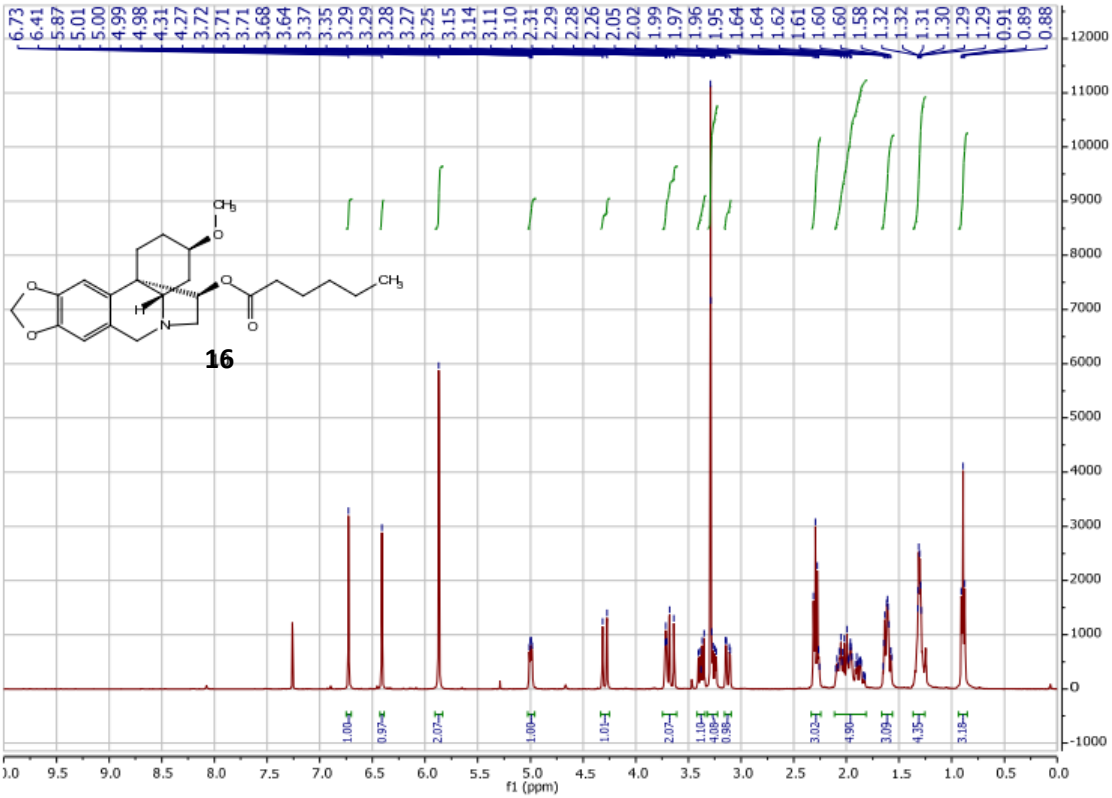
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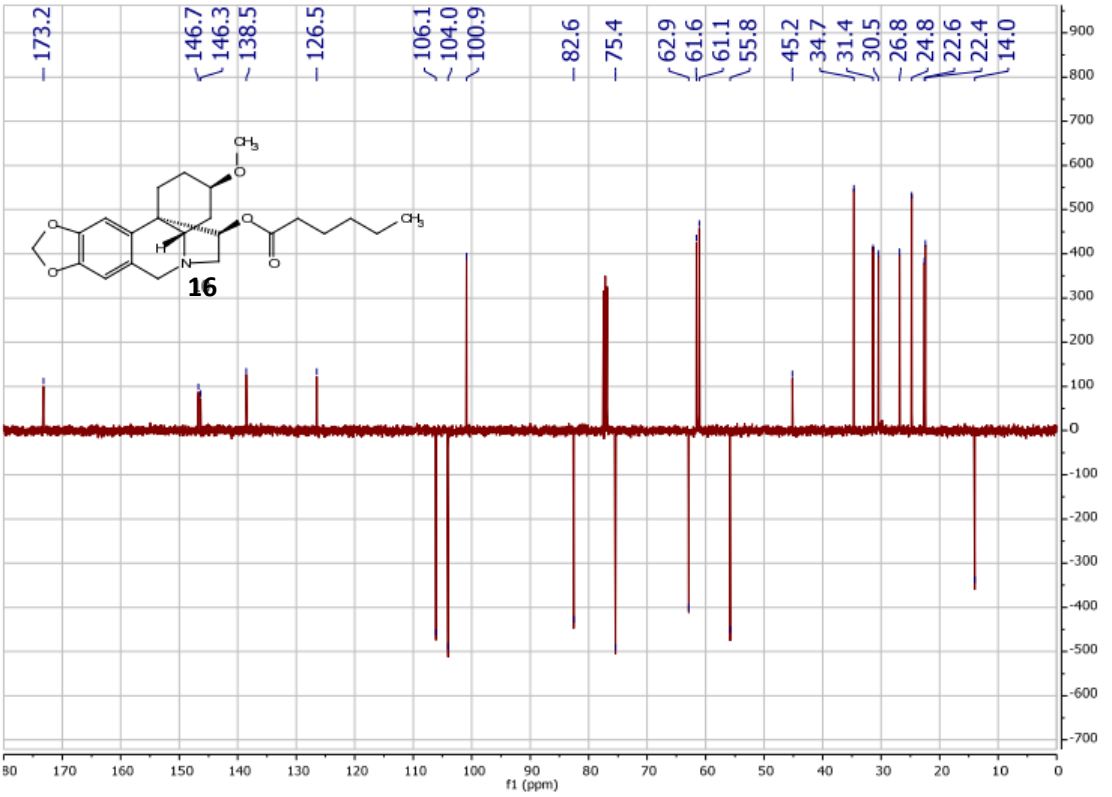
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Appendix – Chapter 2

Publication

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Synthesis of zephycandidine A from haemanthamine

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ABSTRACT

Zephycandidine A the first naturally occurring imidazo[1,2–*f*]phenanthridine alkaloid, isolated from *Zephyranthes candida* (*Amaryllidaceae*) has been prepared in three steps from the naturally occurring alkaloid haemanthamine.

Introduction

The isolation of zephycandidine A **1** in 2016 [1] from *Zephyranthes candida* (*Amaryllidaceae*) represented the first report of a natural product containing an imidazo[1,2–*f*]phenanthridine nucleus. The structure of **1** was elucidated by a combination of spectroscopic analysis and predictive NMR calculations (Fig. 1).

This metabolite exhibited significant cytotoxicity against five cancer cell lines, together with the ability to induce apoptosis in leukaemia cells and displayed acetylcholinesterase (AChE) inhibitory activity. Our own interest in alkaloids from *Amaryllidaceae* lies in the isolation of alkaloids from species of daffodils and particularly with the use of waste-stream material generated from the commercial isolation of galanthamine. Galanthamine is currently used in the early stage treatment of Alzheimer's disease and similarly works by inhibiting acetylcholinesterase (AChE) [2].

Discussion

We were particularly interested in zephycandidine A **1** due to its structural similarity to the known [3] phenanthridine alkaloid trispheridine **2**. This metabolite was isolated [4] from *Zephyranthes candida*, and has been the topic of total synthesis by several groups [5]. We were particularly interested in the early report [6] of Warren and Wright who demonstrated that pyrolysis of the alkaloid haemanthamine **3** on a small scale led to the formation of **2** in good yield. We theorised that trispheridine **2** might be converted into zephycandidine A **1** and indeed the one-pot synthesis of imidazo[1,2–*f*]phenanthridine from phenanthridine reported by Cronin and co-workers [7] was suggested as a potential synthetic method by the group that isolated **1** [1].

Access to daffodil waste-stream material from the isolation of galanthamine from Carlton daffodils [8] allowed us to access large quantities (>100 g) of haemanthamine **3**. Haemanthamine **3** was obtained by basification and extraction of an aqueous plant extract followed by recrystallization of the mixed alkaloids obtained from acetone.

We attempted to repeat the work of Warren and Wright [6] (Scheme 1) who reported that by mixing **3** with powdered zinc and heating the mixture at an unspecified temperature, a high yield of **2** was obtained. In our hands, results on a small scale (100–200 mg of **3** and 3–40 equiv. of Zn dust) were not encouraging. These gave either no conversion at lower temperatures (100–150 °C), partial conversion at 150–190 °C or complete decomposition at higher temperatures (195–220 °C). Reactions on a larger scale suffered from similar problems in that heating over commercially available zinc powder led to a poor conversion at 180–195 °C (Table 1, entries 1–4) whilst heating at 210–220 °C led to near complete decomposition. (Table 1, entry 5). Performing the reaction in air (Entry 1) or under nitrogen (Entry 2) or partial vacuum (Entry 3) had no apparent effects. In these cases haemanthamine **3** was dispersed onto the zinc by evaporation from a methanol solution. Variations in the equivalents of zinc used were also not

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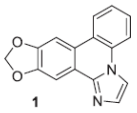
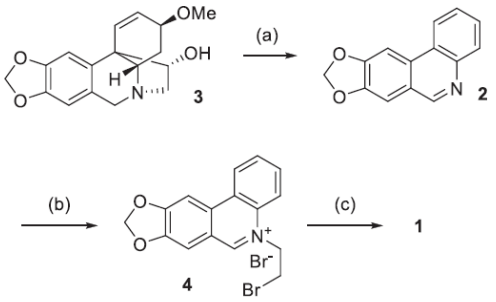


Fig. 1. Zephycandidine A **1**.



Scheme 1. Synthesis of Zephycandidine A **1**. Reagents and conditions: (a) Heat, 190–195 °C, 24 h (see Table 1), 20% (99% based on recovered **3**. (b) 1,2-dibromoethane, 90 °C, 7 days. (c) (i) NH_3 (liquid), −78 °C to −33 °C, 1 h. (ii) MnO_2, $NaCO_3$, −78 °C, 1 h, then overnight warmed to rt. (iii) Toluene, reflux, 3 h, 54% (over 2 steps).

effective in improving the yield (Table 1, entries 1–3). Additionally, the use of freshly activated zinc powder (Table 1, entries 6–7) gave little improvement; in these cases **3** was mixed with the zinc and ground to a fine powder. Considering a possible mechanism for this transformation (Scheme 1) we can visualise a fragmentation of **3** leading to imine **5**, methanol and acetaldehyde enol. Tautomerisation of **5** leads to **6** which upon oxidation, possibly by acetaldehyde, leads to compound **2** (Fig. 2).

This mechanism suggests that the zinc is superfluous to this reaction and we repeated the pyrolysis reaction in its absence. Thus, heating **3** in a sealed tube at 190–195 °C for 24 h led to the formation of **2** in 20% yield together with recovered starting material in 79% yield (Table 1, entry 6). Repeating the pyrolysis for a longer time did not lead to a higher yield as considerable darkening of the reaction was observed after 2 d and complete decomposition was observed after 5 d. We dispersed **3** on sand to prevent direct contact with the oil bath and repeated the pyrolysis but again obtained a lower yield (Entry 9). We next performed pyrolysis reactions in decalin at various temperatures and found no reaction at 175–180 °C over 5 d (Entry 10) and partial conversions at 180–185 °C (Entry 11) and at 190–195 °C (Entry 12) over 7 d. In the latter two cases decomposition was apparent and the amount of recovered starting material low. We also performed a reaction in which we added acetaldehyde as a potential oxidant (Entry 13), however this gave similar yields and levels of decomposition. Whilst these reactions did not give the optimal conversion of haemanthamine **3** into trispheridine **2** previously reported [6], heating **3** under the conditions of entry 8 followed by recycling of the recovered starting material gave reasonable quantities of **2** for the synthesis of **4**.

On heating **2** with freshly distilled 1,2-dibromoethane over 7 days a precipitate formed that was removed periodically to give **4** as an amorphous solid. The material yield for this process was good, however the product was contaminated with significant amounts (*ca.* 10–20%) of what was thought to be the hydrobromide salt of the starting material **2**. These two compounds were difficult to separate and only small quantities of pure **4** could be obtained by trituration in methanol. This by-product was thought to arise from decomposition of the reaction solvent leading to the formation of HBr. We attempted to prepare the salt at a lower temperature over a longer time, however this did not alleviate the problem. We decided to react this mixture under the conditions reported by Cronin and co-workers [7] and thus it was added to liquid ammonia at −78 °C and stirred until completely dissolved. The mixture was then allowed to reflux (−33 °C) for 5 h, cooled to −78 °C and an excess of MnO_2 and Na_2CO_3 added. After warming to −33 °C over 1 h, the reaction was allowed to warm to rt overnight to evaporate the ammonia. At this point the residue was suspended in toluene and heated at reflux for 3 h. After filtration and evaporation, purification by silica gel chromatography gave zephycandidine A **1** as an off white solid in 54% yield together with the recovery of unreacted **2** from the previous step (Scheme 1).

Synthetic zephycandidine A **1** gave NMR data close to that reported in the literature [1] when obtained at 400 MHz. This data was obtained in CD_3OD solution as used in the literature but due to varying amounts of HOD in the sample significant variations in chemical shift on standing and on reanalysis were observed. This might possibly have been due to small variations in the pH of the sample solution. Additionally, as **1** was only sparingly soluble in CD_3OD we observed that without precise control of concentration and temperature very small variation in chemical shift occurred between different experiments. Reanalysis was subsequently performed at higher field (700 MHz) and the assignment of resonances from the proton and carbon NMR spectra for **1** are given in Table 2. The data agreed with the data reported previously

Table 1
Preparation of trispheridine **2**.

Entry	Scale/(mmol)	Temp./°C	Zn/equiv.	Time/h	Yield **2**[i]/%	Recovered **3**/%	Decalin/mL	Sealed
1	16.6	180–90	36	3	10 (24)	59	0	No
2	19.1	190–95	72	24	4 (8)	52	0	No
3	6.6	190–95	185	24	11 (9)	55	0	No
4	16.8	190–95	72	24	11 (22)	50	0	No
5	16.9	210–20	72	7	7	0[ii]	0	No
6	8.3	190–95	75[iii]	24	11 (14)	22	0	No
7	8.3	160–90	75[iii]	48	10 (13)	25	0	No
8	1.7	190–95	0	24	**20 (99)**	80	0	Yes
9	5.0	190–95	0[iv]	24	11 (39)	73	0	Yes
10	6.6	175–80	0	120	0	85	2	Yes
11	6.6	180–85	0	168	8 (15)	46	2	Yes
12	6.6	190–95	0	168	19 (22)	14	2	Yes
13	6.6	190–95	0[v]	24	13 (39)	33	2	Yes

i) Yields in brackets based on recovered **3**. ii) Considerable decomposition occurred. iii) Using freshly activated zinc. iv) **3** was dispersed on sand. v) 2 equiv. of acetaldehyde was added.

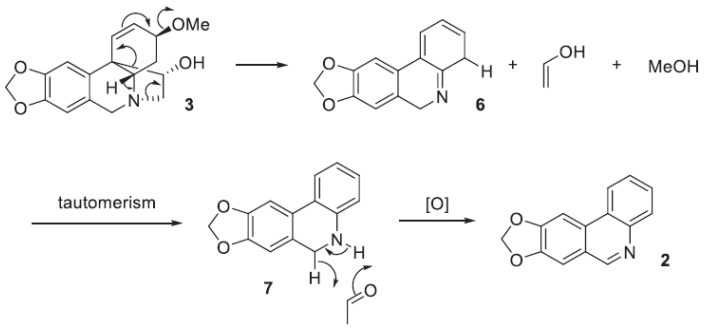


Fig. 2. Possible mechanism for the formation of **2**.

Table 2
Assignment of the NMR resonances for zephycandidine A **1**.

Position	^{13}C/ppm	^{1}H/ppm	J/Hz	Comments
1	125.21	8.39	dddd, 8.1, 1.2, 0.6, 0.6	Couplings to protons at C2, C3, C4 and C10
2	126.6	7.53	ddd, 8.1; 7.2; 1.2	
3	129.6	7.64	ddd, 8.2; 7.2; 1.2	
4	117.3	8.09	dd, 8.2; 1.2, 0.6	NOESY correlates protons at C4 and C11
4a	132.1			
6	143.6			
6a	119.5			HMBC correlates carbon at C6a to proton at C10
7	102.9	7.81	d, 0.6	
8	150.5			
9	151.4			
10	102.8	7.90	dd, 0.6, 0.6	
10a	125.23			
10b	122.9			
11	113.7	8.28	d, 1.5	NOESY correlates protons at C11 and C4
12	131.2	7.50	d, 1.5	
CH_2	103.5	6.15	d, 0.5	This residual dipolar coupling is a result of some molecules of **1** having preferential orientation in solution at 16.44 T and it has been documented for aromatic molecules before [8].

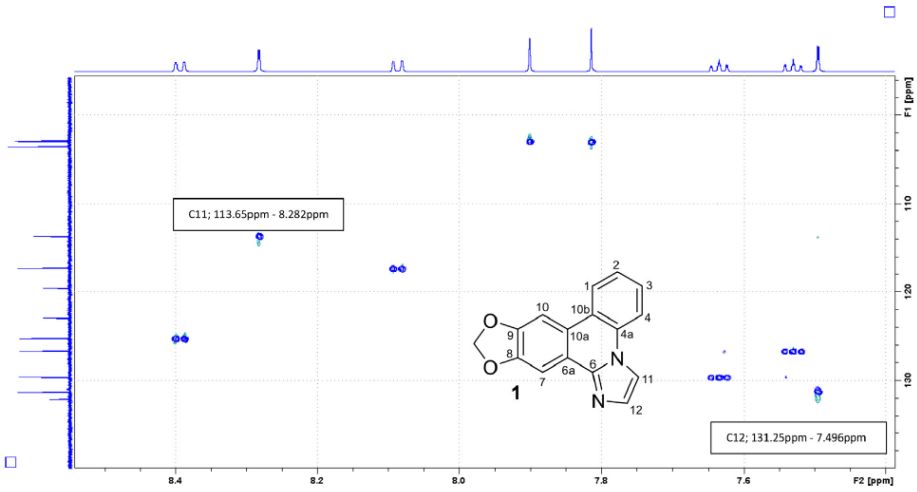


Fig. 3. Section of HSQC spectrum with the marked correlations at C11 and C12 positions.

[1] except for the protons at positions C-11 and C-12 which were misassigned in the original report. The HSQC spectrum of **1** indicates a clear correlation between the chemical shift of the signal at 113.7 ppm (C-11) and the proton at 8.28 ppm and the signal at 131.3 ppm (C-12) and the proton at 7.50 ppm (Fig. 3). Synthetic **1** was fully soluble in a 10:1 mixture of $CDCl_3$ and CD_3OD and gave consistent NMR data. Full spectroscopic data and a comparison of NMR data for synthetic and natural **1** is available in the ESI.

Conclusion

In conclusion, the *Amaryllidaceae* alkaloid zephycandidine A **1** has been independently synthesised for the first time from the alkaloid haemanthamine **3** *via* the alkaloid trispheridine **2**, confirming the proposed structure.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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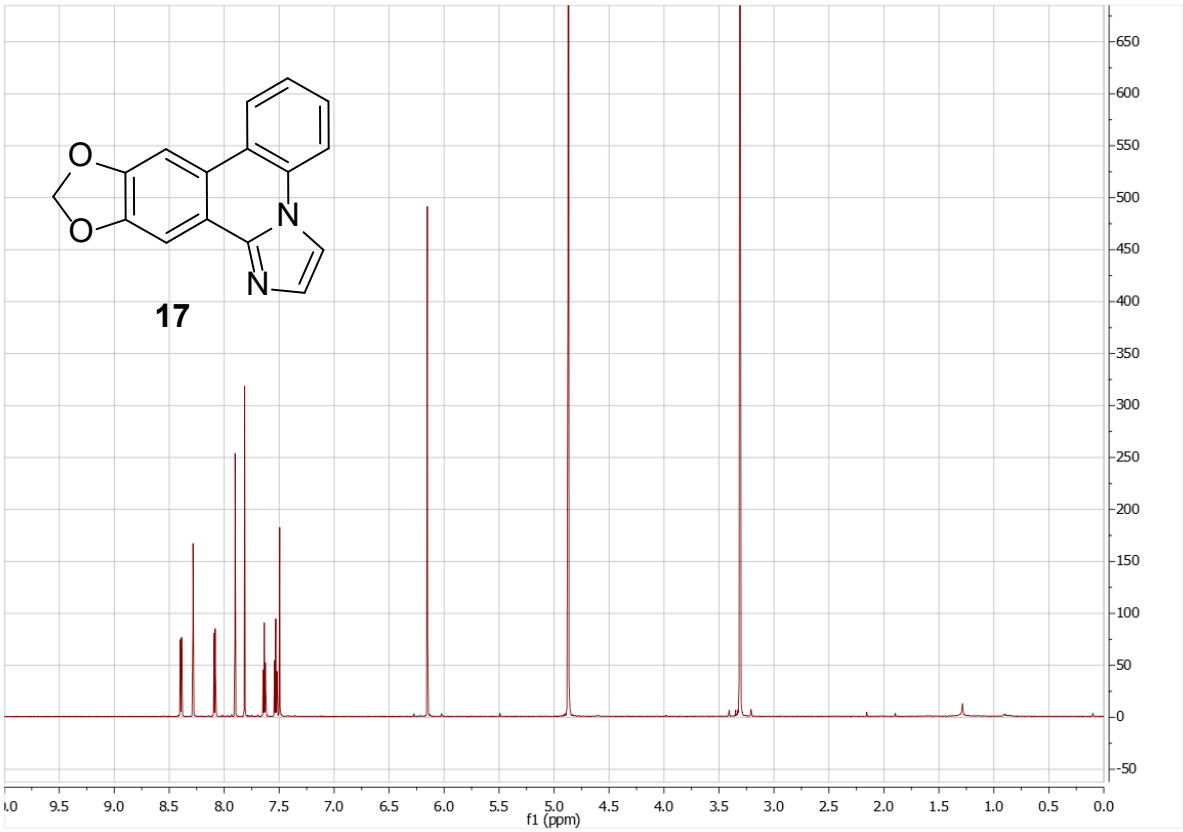
Appendix A. Supplementary data

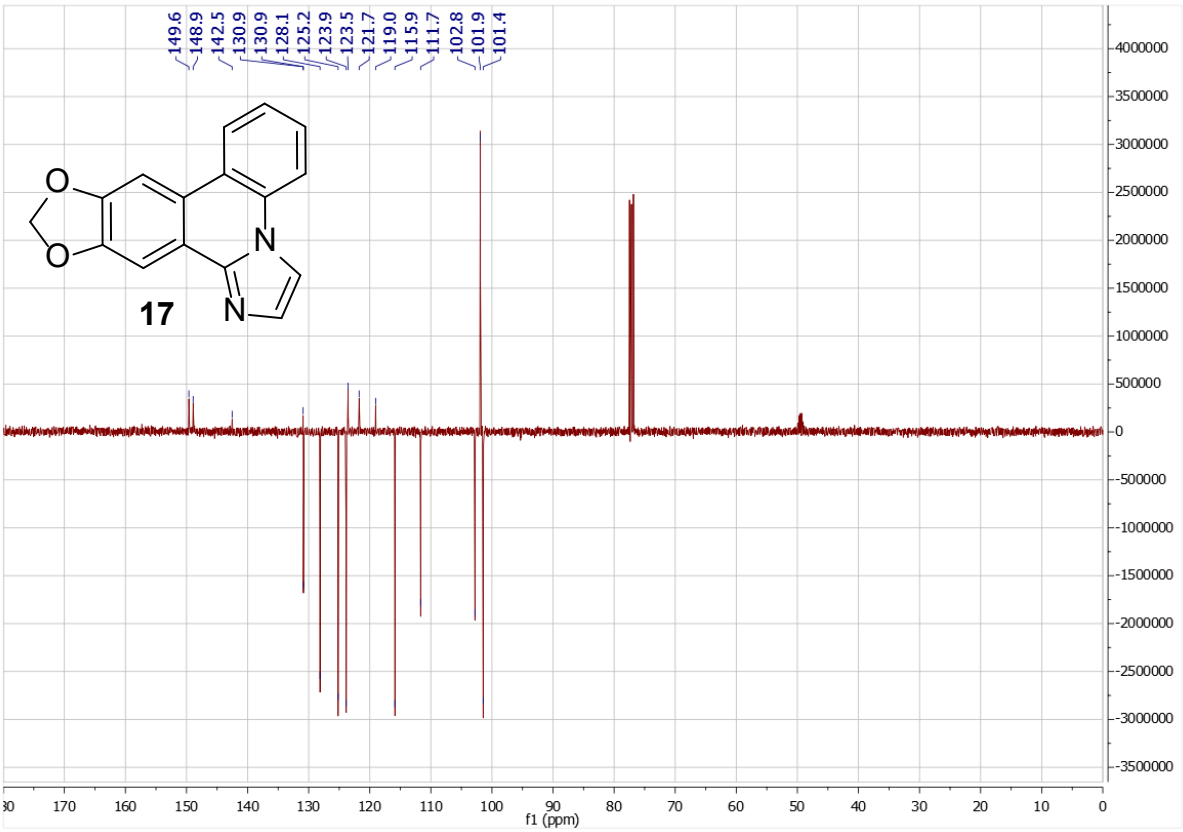
Supplementary data to this article can be found online at https://doi.org/10.1016/j.ultsonch.2019.104640.

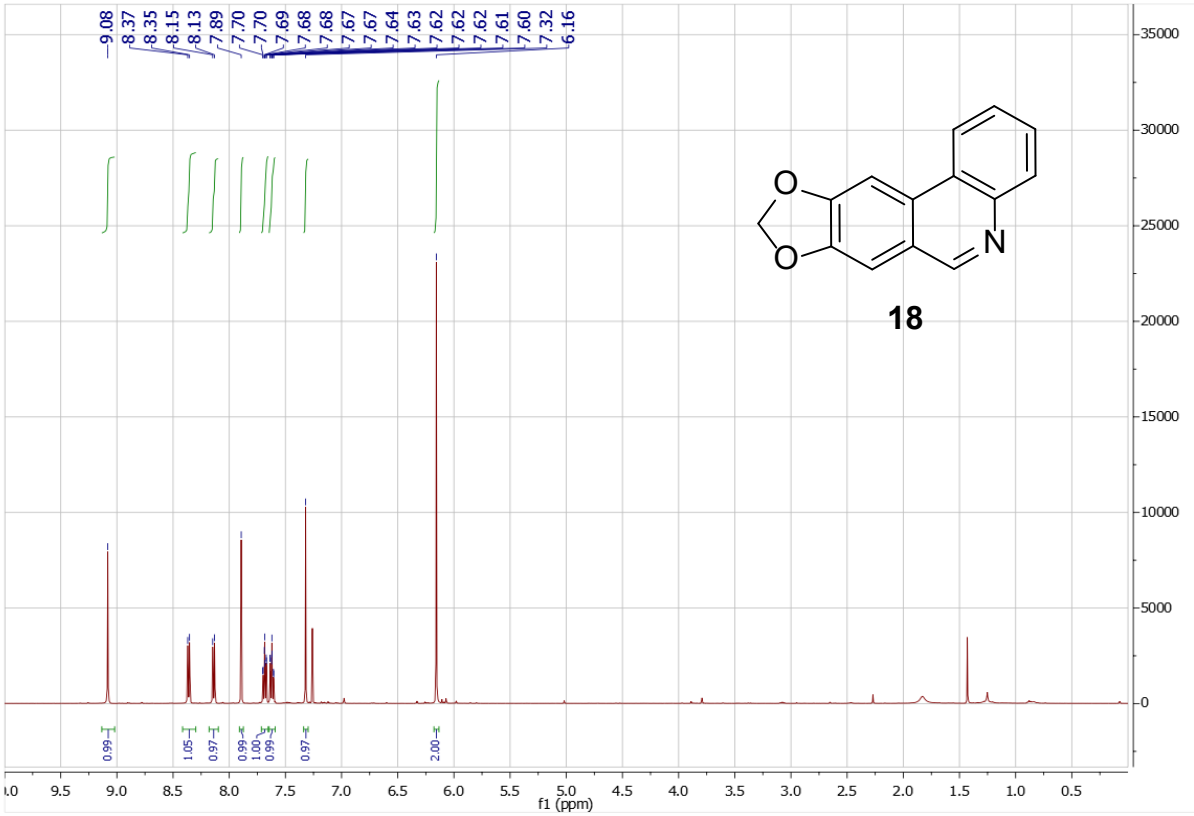
References

[1] G. Zhan, X. Qu, J. Liu, Q. Tong, L. Zhou, B. Sun, G. Yao, Sci Rep. 6 (2016) 33990.
[2] M.-J.R. Howes, N.S.L. Perry, P. Houghton, J. Phytother. Res. 17 (2003) 1–18.
[3] S. Ghosal, K.S. Saini, S. Razdan, Phytochemistry 24 (1985) 2141–2156.
[4] Z. Luo, F. Wang, J. Zhang, X. Li, M. Zhang, X. Hao, Y. Xue, Y. Li, F.D. Horgen, G. Yao, Y. Zhang, J. Nat. Prod. 75 (2012) 2113–2120.
[5] a) T. Kumemura, T. Choshi, J. Yukawa, A. Hirose, J. Nobuhiro, S. Hibino, Heterocycles 66 (2005) 87–90;
b) M.E. Budén, V.B. Dorn, M. Gamba, A.B. Pierini, R.A. Rossi, J. Org. Chem. 75 (2010) 2206–2218;
c) S. De, S. Mishra, B.N. Kakde, D. Dey, A. Bisai, J. Org. Chem. 78 (2013) 7823–7844;
d) A. Borah, P. Gogoi, Eur. J. Org. Chem. (2016) 2200–2206;
e) T. Harayama, H. Akamatsu, K. Okamura, T. Miyagoe, T. Akiyama, H. Abea, Y. Takeuchia, J. Chem. Soc., Perkin Trans. 1 (2001) 523–528.
[6] F.L. Warren, W.G. Wright, J. Chem. Soc. (1958) 4696–4700.
[7] A.D.C. Parenty, K.M. Guthrie, Y.-F. Song, L.V. Smith, E. Burkholder, L. Cronin, Chem. Commun. (2006,) 1194–1196.
[8] a) P.C.M. van Zijl, B.H. Ruessink, J. Bulthuis, C. MacLean, Acc. Chem. Res. 17 (1984) 172–180;
b) F.A.L. Anet, M. Kopelevich, J. Am. Chem. Soc. 108 (1986) 1354–1355;
c) L.B. Alemany, A. Gonzalez, W.E. Billups, M.R. Willcott, E. Ezell, E. Gozansky, J. Org. Chem. 62 (1997) 5771–5779.

NMR spectra of compounds 17-19



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6.16
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1.00
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2.00
O
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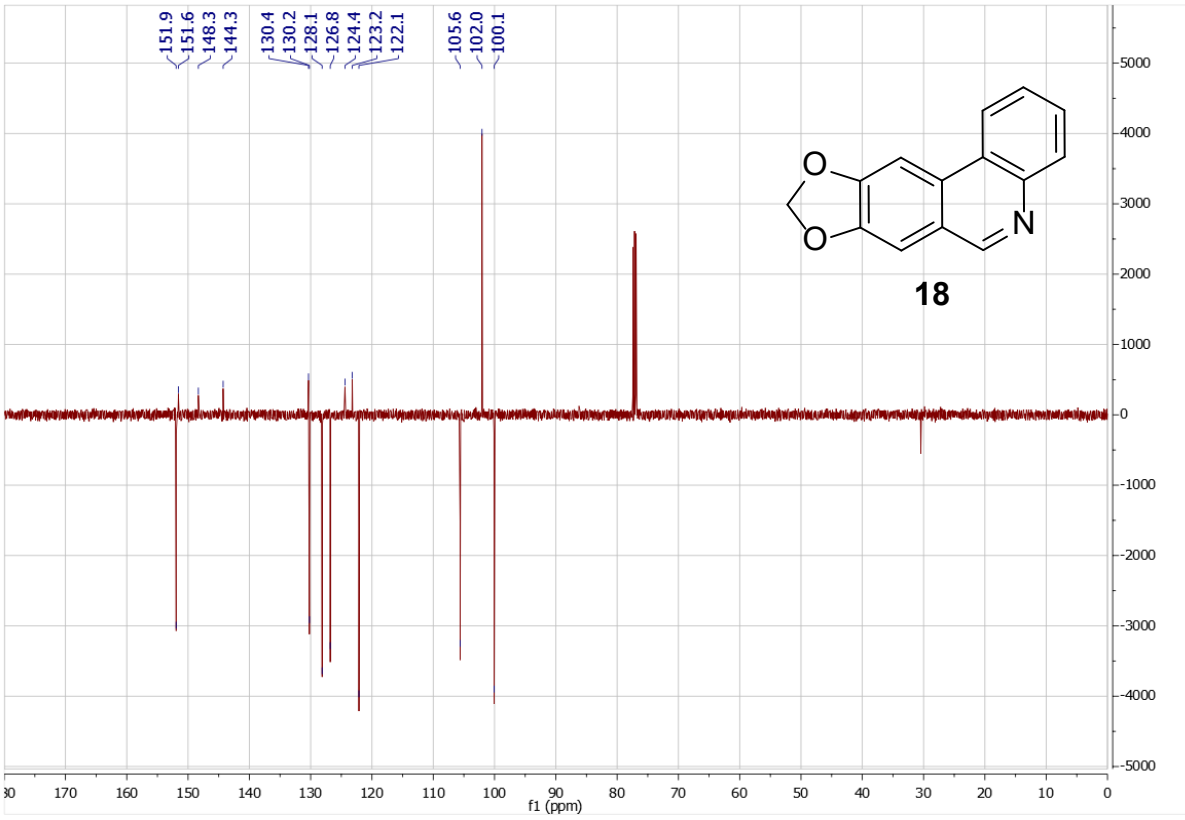
18
f1 (ppm)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100.1
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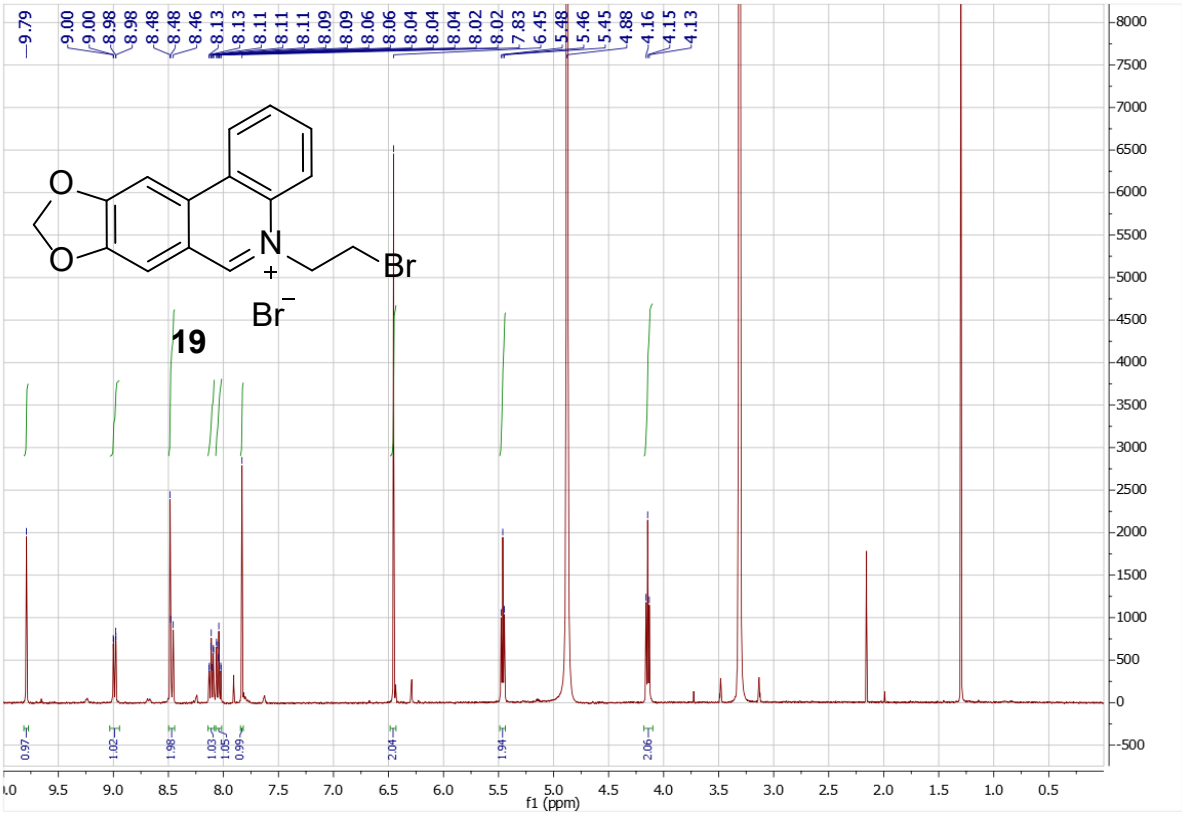
f1 (ppm)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7.83
6.45
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O
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Br
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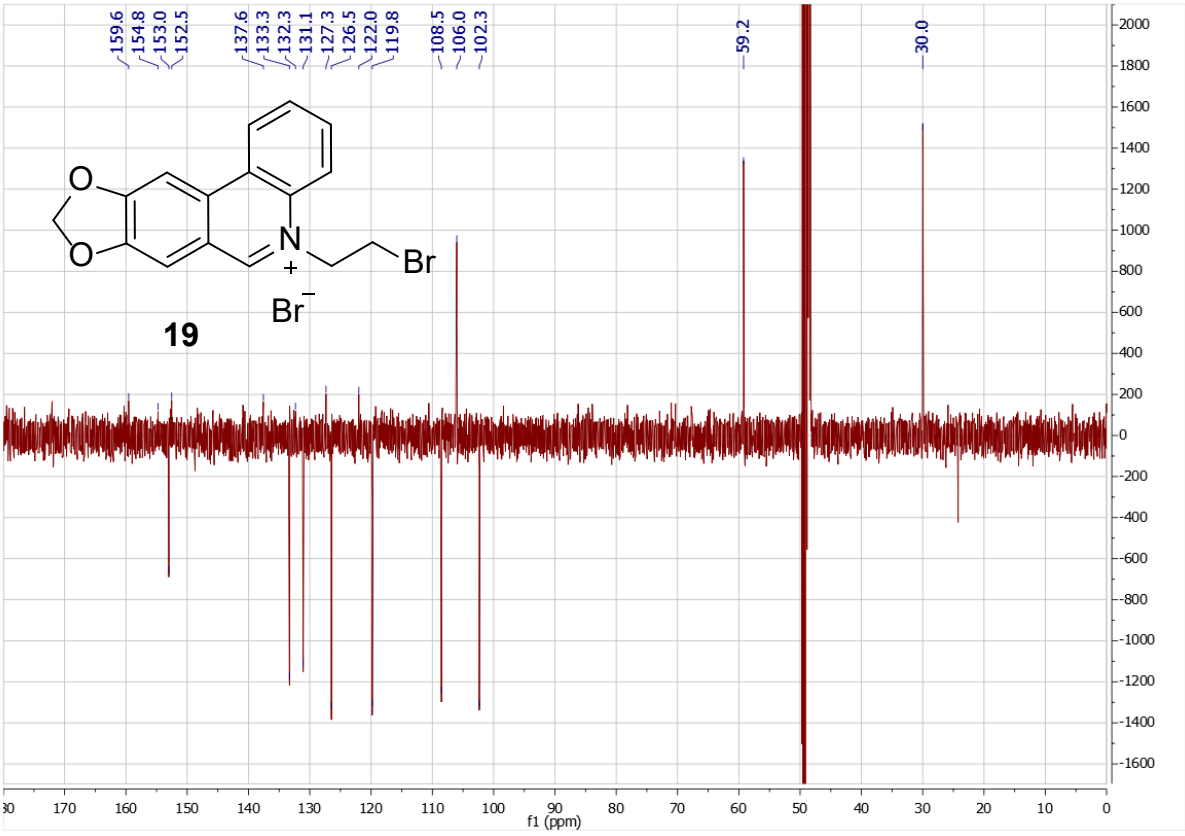
19
f1 (ppm)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106.0
102.3
59.2
30.0
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O
N
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19
f1 (ppm)

Appendix – Chapter 3

Publication

PLOS NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Quorum sensing N-Acyl homoserine lactones are a new class of anti-schistosomal

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Abstract

Background

Schistosomiasis is a prevalent neglected tropical disease that affects approximately 300 million people worldwide. Its treatment is through a single class chemotherapy, praziquantel. Concerns surrounding the emergence of praziquantel insensitivity have led to a need for developing novel anthelmintics.

Methodology/Principle findings

Through evaluating and screening fourteen compounds (initially developed for anti-cancer and anti-viral projects) against *Schistosoma mansoni*, one of three species responsible for most cases of human schistosomiasis, a racemic N-acyl homoserine (**1**) demonstrated good efficacy against all intra mammalian lifecycle stages including schistosomula (EC_{50} = 4.7 μM), juvenile worms (EC_{50} = 4.3 μM) and adult worms (EC_{50} = 8.3 μM). To begin exploring structural activity relationships, a further 8 analogues of this compound were generated, including individual (*R*)- and (*S*)- enantiomers. Upon anti-schistosomal screening of these analogues, the (*R*)- enantiomer retained activity, whereas the (*S*)- lost activity. Furthermore, modification of the lactone ring to a thiolactone ring (**3**) improved potency against schistosomula (EC_{50} = 2.1 μM), juvenile worms (EC_{50} = 0.5 μM) and adult worms (EC_{50} = 4.8 μM). As the effective racemic parent compound is structurally similar to quorum sensing signaling peptides used by bacteria, further evaluation of its effect (along with its stereoisomers and the thiolactone analogues) against $Gram^+$ (*Staphylococcus aureus*) and $Gram^-$ (*Escherichia coli*) species was conducted. While some activity was observed against both $Gram^+$ and $Gram^-$ bacteria species for the racemic compound **1** (MIC 125 mg/L), the (*R*) stereoisomer had better activity (125 mg/L) than the (*S*) (>125mg/L). However, the greatest antimicrobial activity (MIC 31.25 mg/L against *S. aureus*) was observed for the thiolactone containing analogue (**3**).

Conclusion/Significance

To the best of our knowledge, this is the first demonstration that N-Acyl homoserines exhibit anthelmintic activities. Furthermore, their additional action on $Gram^+$ bacteria opens a new avenue for exploring these molecules more broadly as part of future anti-infective initiatives.

Competing interests: The authors have declared that no competing interests exist.

Author summary

Schistosomiasis, caused by infection with blood fluke schistosomes, is a neglected tropical disease that negatively impacts the lives of approximately 300 million people worldwide. In the absence of a vaccine, it is currently controlled by a single drug, Praziquantel (PZQ). Although incredibly valuable in controlling disease burden, PZQ-mediated chemotherapy is ineffective against juvenile worms and may not be sustainable should resistance develop. The need to identify an alternative or combinatorial drug is, therefore, a priority in contributing to the control of this parasitic disease into the 21st century. In this study, we have identified a new class of anthelmintic, N-acyl homoserine lactones, which are normally used by bacteria for quorum sensing and population density control. The tested N-acyl homoserine lactones were active against all intra-human schistosome lifecycle stages, in particular, when a thiolactone modification to the core N-acyl homoserine ring was made. Interestingly, these N-acyl homoserine lactones also displayed antimicrobial activities against Gram$^+$ *Staphylococcus aureus*. By demonstrating broad activities against schistosomes and bacteria exemplars, this study identified a potential route for the further development of a new anti-infective class.

Introduction

The development of drug resistant prokaryotic and eukaryotic pathogens is of great concern for the sustainable control of both human and animal diseases; therefore, the need for new anti-infectives is a global health priority. One notorious group of difficult to control pathogens are those that cause the Neglected Tropical Diseases (NTDs). In total, 17 parasites/microbes and their related infections are now identified as responsible for most NTDs worldwide [1], with schistosomiasis, leishmaniasis and soil-transmitted helminthiasis causing significant disability adjusted life years lost per annum [2]. As vaccines are unavailable for the prevention of most NTDs, a small number of chemotherapies remain the primary means of global control. However, drug resistance or reduced susceptibility to these limited drug classes has been reported for NTD-causing bacteria [3], fungi [4], helminths [5, 6] and protozoa [7, 8]. These chemotherapy limitations have prompted an urgent need for research into the development of new anti-infective agents. Nevertheless, funding to support this agenda is unlikely to originate from the pharmaceutical sector due to the lack of financial returns associated with controlling diseases predominantly affecting low to middle-income countries [9–11]. Thus, philanthropic and public organisations are often driving the majority of new anti-infective initiatives targeting the NTDs with derived funding supporting research conducted in higher education or research institutes.

One particular debilitating NTD, schistosomiasis, affects over 300 million people worldwide [12] and is predominantly caused by infection with three *Schistosoma* species [13]. Therapeutic treatment involves praziquantel (PZQ) as the frontline control strategy. However, PZQ is ineffective against the juvenile stage of the parasite *in vivo*, which necessitates repeat administrations to reach maximal efficacy in endemic populations [14]. Furthermore, PZQ is currently produced as a racemic mixture and only the (*R*)—enantiomer is active; the (*S*)—enantiomer contributes to some of the side effects including bitter taste and non-compliance in the young [15]. These drug-related limitations, together with a constant fear of PZQ resistance developing, has fuelled investigations into the identification of PZQ replacement or combinatorial anti-schistosomal drugs.

Towards this goal, our group has recently identified several diverse starting points for anti-schistosomal drug discovery. These include diterpenoids [16, 17], triterpenoids [18], and epigenetic probes/inhibitors [19, 20]. While the primary focus of these investigations evaluated compound-induced activity on *Schistosoma mansoni* (the schistosome species responsible for both Old and New world schistosomiasis), parallel studies were also conducted to quantify anti-infective activities against other NTD-causing pathogens including *Fasciola hepatica* (liver fluke) [16–18] or NTD models such as *Mycobacterium smegmatis* (related to *Mycobacterium leprae*) [21].

In this present study, we assessed the anti-schistosomal activity of 14 in house prepared synthetic compounds (or intermediate analogues of these compounds). As the most effective anti-schistosomal compounds were structurally similar to N-acyl homoserines, a class of signalling molecule involved in bacterial quorum sensing and population density control [22], we additionally investigated their anti-microbial activity. Amongst the compounds tested, one demonstrated moderate activity against Gram$^+$ (*Staphylococcus aureus*) bacteria. Our collective results demonstrate that N-acyl homoserines represent a new class of anthelmintics with additional activity against bacteria. Further development of these molecules could be pursued as promising new chemotherapeutics for schistosomiasis and other NTDs.

Materials and methods

Ethics statement

All procedures performed on mice (project licenses 40/3700 and P3B8C46FD) adhered to the United Kingdom Home Office Animals (Scientific Procedures) Act of 1986 as well as the European Union Animals Directive 2010/63/EU and were approved by Aberystwyth University's (AU) Animal Welfare and Ethical Review Bodies (AWERB).

Synthetic methodology

Lactones (compounds **1–9**) were prepared by the reaction of the lactone/thiolactone, with the required acyl halide in the presence of NEt_3 in chloroform or potassium carbonate in a two phase water/chloroform mix (Scheme 1 in S1 Protocol). Yields and conditions are shown in Table 1 and full synthetic and spectroscopic details are found in S1 Protocol.

Compound storage and handling

All compounds were solubilised in DMSO (Fisher Scientific, UK) to a stock concentration of 10 mM and stored at -20˚C until required. Positive controls for *S. mansoni* screens included PZQ (Sigma-Aldrich, UK) and auranofin (Sigma-Aldrich, UK), which were also treated in the same manner as the test compounds.

Screening of *S. mansoni* schistosomula

Biomphalaria glabrata (NMRI and the previously described pigmented strains [23]) snails infected with *S. mansoni* (Puerto Rican strain) were shed for 2 hrs under light at 26 °C. Cercariae were collected, mechanically transformed into schistosomula [24] and subsequently prepared for high throughput screening (HTS) on the Roboworm platform as previously described [17]. Schistosomula were cultured in Basch Media (composed of BME media, 10% M169 media, 0.5x MEM vitamins, 10% Schneider's insect media, 15.6mM Hepes, 5% heat inactivated FBS, 2x Antibiotic/anti-mycotic (Sigma-Aldrich, UK) solution (final concentrations shown)).

Table 1. Preparatory summary of compounds 1–9.

Compound	X	Z	R	*	#	Method	Yield	Mp/°C
1	Br	O	H	Racemic	NA	A	75%	95–97
2	Br	O	Me	Racemic	Racemic	A	33%	157–69
3	Br	S	H	Racemic	NA	B	40%	111–3
4	Br	S	Me	Racemic	Racemic	B	73%	125–28
5	Br	O	H	*R*	NA	B	25%	130–3
6	Br	O	H	*S*	NA	B	14%	130–3
7	Cl	O	H	Racemic	NA	A	68%	111–5
8	Cl	S	H	Racemic	NA	B	61%	124–7
9	Cl	O	Me	Racemic	Racemic	A	65%	148–50

The symbols
"*" and
"#" refer to the absolute configuration at the carbon centres indicated in scheme 1.
NA = Not applicable
Z = O, S, R = H, Me.

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Compounds were initially tested at a final concentration of 10 μM and those that were active were further titrated at concentrations of 10, 5, 2.5, 1.25 and 0.625 μM. EC_{50} values were calculated from the titrated concentrations by non-linear regression, after log transformation of concentrations and data normalization using GraphPad Prism 7.02.

Screening of adult *S. mansoni* blood flukes (7-week worms)

Adult *S. mansoni* parasites were recovered by hepatic portal vein perfusion from TO outbred mice (HsdOla:TO, Tuck-Ordinary, Envigo, UK) that were percutaneously infected seven weeks earlier with 180 cercariae. Three adult worm pairs per well, in duplicate, were transferred into 48 well plates (Fisher Scientific, Loughborough, UK) and cultured at 37 °C in an atmosphere containing 5% CO_2 in DMEM (Gibco, Paisley, UK) containing 10% v/v HEPES, 10% v/v Foetal Bovine Serum (FBS), 0.7% v/v 200 mM L-Glutamine and 1X v/v penicillin-streptomycin. Worms were dosed with test compounds at 20 μM, 10 μM, 5 μM, 2.5 μM, 1.25 μM and 0.625 μM (in 0.2% DMSO) for 72 hr. Adult worms were scored manually at 72 hr using the WHO-TDR metric scoring system as described previously [25]. Dose response curves and EC_{50} values were obtained by non-linear regression, after log transformation of concentrations and data normalization using GraphPad Prism 7.02. At 72 hr, the medium from each well was also collected, centrifuged at 350 x *g* for 2 min. Afterwards, the supernatant was removed, and the remaining egg pellet re-suspended in 10% v/v formalin. Eggs that were oval and contained a fully formed lateral spine were subsequently counted.

Screening of juvenile *S. mansoni* blood flukes (3-week worms)

Juvenile *S. mansoni* parasites were recovered via hepatic portal vein perfusion from TO outbred mice (HsdOla:TO, Tuck-Ordinary, Envigo, UK) that were infected percutaneously three weeks earlier with 4000 cercariae. Preparation and centrifugation of juvenile worms have been described previously [18]. Briefly, juvenile worms (n = 13–33 individuals/well) in 200 μl of a 96-well tissue culture plate were co-cultured with compounds (15 μM, 7.5 μM, 3.75 μM, 1.83 μM, 0.94 μM and 0.47 μM (in 1.25% DMSO) in DMEM (Gibco, Paisley, UK) supplemented with 10% v/v HEPES (Sigma-Aldrich, Gillingham, UK), 10% v/v FBS (Gibco, Paisley, UK), 0.7% v/v 200 mM L-Glutamine (Gibco, Paisley, UK) and 1X v/v penicillin-streptomycin

(Fisher Scientific, UK). Positive control wells included either PZQ or auranofin (15 μM in 1.25% DMSO) whereas negative wells included DMSO (1.25%). Parasites were incubated at 37 °C in an atmosphere containing 5% CO_2 for 72 hr at which time worm motility was scored between 0 and 4: 0 = dead, 1 = movement of the suckers only and slight contraction of the body, 2 = movement at the anterior and posterior regions only, 3 = full body movement but sluggish and 4 = normal movement. After motility quantification, 2 μg/mL of PI was added to each well and the plate returned to 37˚C, 5% CO_2 for 15 minutes [26]. Each well was subsequently imaged on the Roboworm platform using brightfield and fluorescent microscopy (excitation wavelength = 580 nm; emission wavelength = 604 nm). The number of PI positive vs PI negative juvenile worms were cross-checked with the motility scores obtained by our scoring matrix, and the data reported as percentage of PI positive across all parasites within the well. EC_{50} values were calculated from the motility scores obtained from the dose response titration (as detailed above) and dose response curves were obtained by non-linear regression, after log transformation of concentrations and data normalization using GraphPad Prism 7.02.

Cell cytotoxicity assays

The cytotoxicity of each compound was assessed on human HepG2 cells as described previously [17]. Briefly, 2 x 10^4 cells/well were seeded in black walled 96-well microtiter plates (Fisher Scientific, Loughborough, UK) and incubated for 24 hr at 37˚C in a humidified atmosphere containing 5% CO_2. To each well, compounds were subsequently added to obtain final concentrations (in 1% DMSO) of 100 μM, 75 μM, 50 μM, 25 μM, 10 μM and 5 μM. Following a further incubation for 24 hr, the MTT assay was performed as previously described [17, 27]. Dose response curves were obtained by non-linear regression, after log transformation of concentrations and data normalization using GraphPad Prism 7.02.

Bacterial growth, minimum inhibitory concentration (MIC) calculation, and EC_{50} determination

S. aureus ATCC 29213 and *E. coli* ATCC 25922 were cultured in Luria-Bertani (LB) medium at 37˚C with aeration at 200 rpm for 24 hr, with all procedures performed in a biosafety level 2 (BSL2) cabinet. Stationary phase cultures were then used for minimum inhibitory concentration (MIC) determination using the broth microdilution method, in fresh LB medium, in a 96-well plate [28]. All compounds were tested in triplicate using an initial bacterial concentration of 5.0 × 10^5 colony forming units (CFU)/mL at a final concentration of 125 mg/L (5% and 2.5% v/v methanol). Compounds with no visible growth at 125 mg/L were further evaluated with progressing dilutions. The MIC was determined as the lowest concentration of a compound at which no growth was visible after 24 hr. Dilutions were repeated in three independent experiments where the optical density (OD_{600}) was measured in a Hidex plate spectrophotometer and absorbance data used for the calculation of an IC_{50} value. The IC_{50} value was obtained from a dose response titration (125–0.09 mg/L). Dose response curves were obtained by non-linear regression, after log transformation of concentrations and data normalization using GraphPad Prism 7.02.

Bioinformatics

The names and structures (SMILES strings) of chemical compounds were identified in PubMed abstracts in June 2019 using the chemistry text-mining software LeadMine v 3.1.2 (NextMove Software Ltd.) [29]. Chemical compounds were identified in all PubMed abstracts containing any one of long list of words relating to schistosomes and anthelmintic/antiparasitic compounds (e.g. 'schistosoma', 'sporocyst', 'sporocysts', 'miracidium', 'miracidia',

'somule', 'somules', 'schistosomula', 'schistosomulum', 'cercariae', 'cercaria', 'schistosome', 'schistosomes', 'antiparasitic', 'antinematodal', 'anthelmintic', etc.). The structures of our screening hits were compared to those of the chemical compounds identified in PubMed abstracts using DataWarrior [30], by using DataWarrior's Similarity Analysis function with its FragFP descriptor.

A map of the quorum sensing pathway in *Pseudomonas aeruginosa* was found using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Through this database, the NCBI Protein IDs and the corresponding amino acid sequences were obtained for LasR (NP_250121), LasI (NP_250123), RhlR (NP_252167), RhlI (NP_252166). These sequences were used as queries for a protein BLAST (BLASTp) search against the *S. mansoni* genome in Wormbase-Parasite [31].

Statistics

All Statistical analyses were conducted using GraphPad Prism 7 software. To determine significant differences amongst population means, a Kruskal-Wallis ANOVA followed by Dunn's multiple comparisons test was used. *p* values are indicated as follows: * <0.05, ** <0.01, *** <0.001.

Results

As part of our anti-infective research activities, a total of 14 synthetic compounds (including some intermediate analogues; S1 Table) were entered into a screening pipeline to identify active molecules (Fig 1).

These 14 compounds (S1 Table) were initially screened against the larval stage of *S. mansoni* (schistosomula) (S1 Fig). Amongst the collection, two compounds negatively affected both schistosomula motility and phenotype metrics at 10 μM (Compound **1** and Compound **E** (S1A Fig)). Subsequent titration of these two compounds demonstrated that Compound **1** was more effective (an average EC_{50} of 4.7 μM for phenotype and motility) than Compound **E** (an average EC_{50} of 5.6 μM for phenotype and motility (S1B Fig)). Due to these initial anti-schistosomula screens identifying compound **1** as being moderately potent, a total of 8 analogues, all containing a lactone ring core structure but differing in functional group modifications, were synthesised to further assess anti-schistosomal activities (S1 Table).

Firstly, in a direct comparison to compound **1**, each of the 8 analogues as well as compound **1** were titrated against the schistosomula stage to assess compound-induced changes to parasite motility and/or phenotype (Fig 2).

Out of the nine compounds titrated, four affected schistosomula motility (44.4%; compounds **1**, **3**, **4** and **5**) (Fig 2A). Compounds **4** and **5** affected the motility of the parasites at both 10 and 5 μM, whereas compound **1** and **3** affected the motility at all concentrations tested. When evaluating compound-mediated alterations of schistosomula phenotypes, five of the nine compounds had an effect (55.6%; compounds **1**, **3**, **4**, **5** and **7**) (Fig 2B). Analogues **4**, **5** and **7** affected the phenotype of the parasites at 10 and 5 μM, compound **1** affected this metric from 10 to 1.25 μM and compound **3** affected the phenotype of the schistosomula at all concentrations tested. Therefore, a substitution of the simple lactone ring (**1**) for a thiolactone ring (**3**) increased comparative anti-schistosomula potency at all concentrations tested. Of particular interest is the comparison of the two enantiomeric forms of Compound **1**. The (*R*)-enantiomer (**5**) affected schistosomula for both phenotype and motility at 10 μM and 5 μM concentrations. However, no anti-schistosomula activity was observed for the (*S*)-enantiomer (**6**), suggesting that stereo-specificity of N-acyl homoserine is critical to structural activity relationships (SAR).

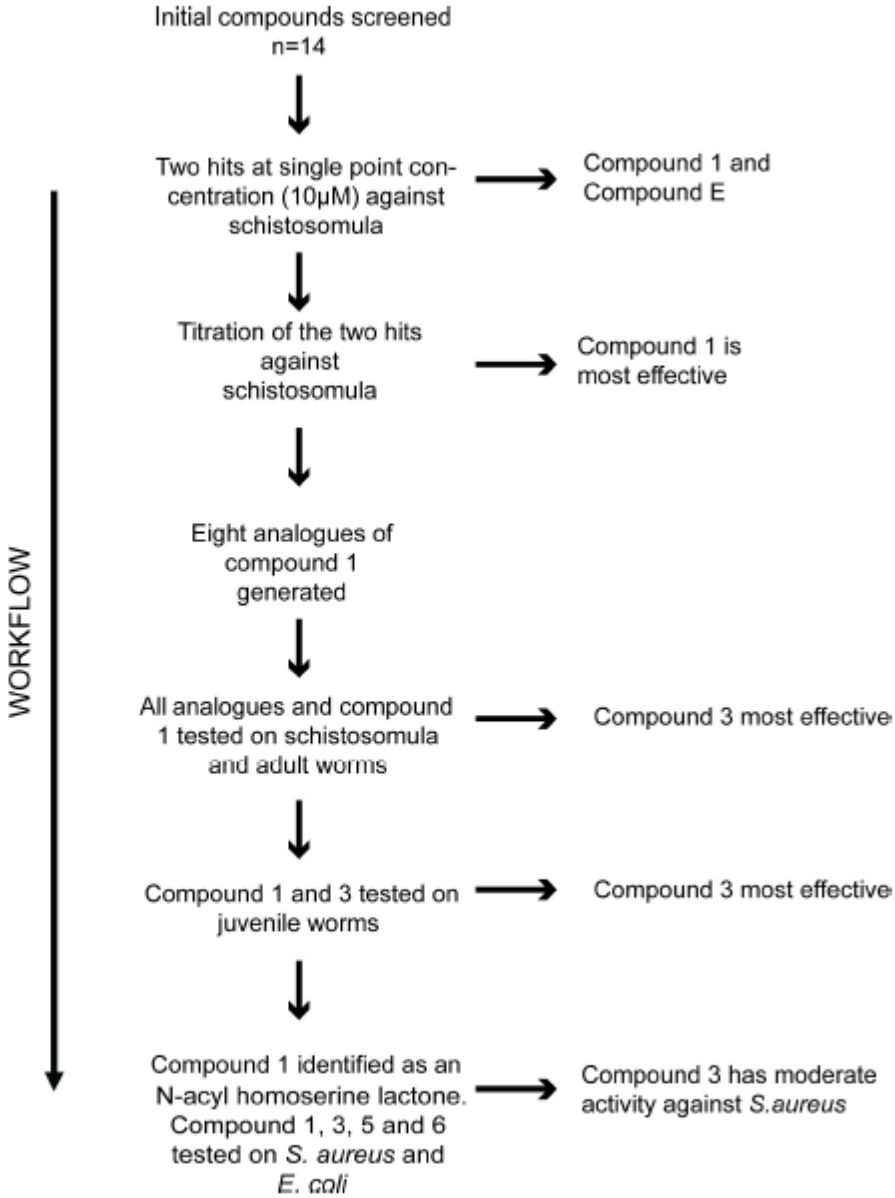

Fig 1. The screening pipeline utilised in this anti-infective study. A total of 14 compounds were initially screened against *S. mansoni* schistosomula at a final concentration of 10 μM. Hits were subsequently subjected to dose response titrations with the most effective anti-schistosomula compound (Compound 1) subsequently being used as a template for the preparation of further derivatives (compounds 2–9). Compounds 2–9 were subsequently subjected to dose response titrations against schistosomula and adult worms. The most

effective compounds (original compound **1** and analogue **3**) were next titrated against juvenile worms. Finally, compounds **1**, **3**, **5** and **6** were additionally titrated against both Gram$^+$ (*S. aureus*) and Gram$^-$ (*E. coli*) bacterial exemplars.

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Due to initial indications that these analogues had varying activities against the schistosomula lifecycle stage (Table 2), the parent compound and its 8 analogues (Compounds **1**–**9**) were subsequently screened against 7-week old adult male and female worms (Fig 3).

Initially, these screens were conducted at 20 μM; any compounds that scored a 1 or 0 for motility at this primary concentration were further titrated until no further effect was observed. For Compound **1**, an EC_{50} for males was estimated to be 8.2 μM and for females 8.3 μM (Fig 3A). Similar to the schistosomula assays, the differences observed in adult parasite motility when treated with the (*R*)- and (*S*)- enantiomers of Compound **1** were striking. The (*R*)-enantiomer (**5**) caused complete immobility at the highest dose of 20 μM; conversely, the (*S*)-enantiomer (**6**) did not have this effect. Estimated EC_{50}s for male and female parasites for these two enantiomers were determined (Table 2), with Compound **5** ((*R*)- enantiomer) having greater activity than Compound **6** ((*S*)- enantiomer).

While the propanamide analogue (**2**) had no activity against the adults, the thiolactone analogue (**3**) had good activity (similar to its effects on schistosomula) with significant reduction in motility observed down to 5 μM; EC_{50}s of 4.2 μM for males and 3.9 μM for females were determined (Table 2). The thiolactone propanamide analogue (**4**) only demonstrated lethality at the highest concentration of 20 μM with an EC_{50} of 10.5 μM and 18.4 μM for males and females respectively (Table 2). No lethality/motility defects were observed for any of the chloro- analogues (Compounds **7**, **8** and **9**).

As morbidity associated with schistosomiasis is caused by egg-induced granuloma formation in tissues and the subsequent development of fibrotic lesions around these granulomas [32], compound-mediated modulation of *in vitro* egg production (as a surrogate for the *in vivo* pathology initiator) was next assessed (Fig 3B). Specifically, media derived from adult worm cultures incubated with compounds that resulted in complete immobility/lethality (compounds **1**, **3**, **4**, and **5**; compound **6** was also included as the (*S*)- stereoisomer of compound **1**) were collected and eggs counted (Fig 3B). In comparison to the negative control DMSO, in which egg counts ranged from 300–594 (Average—408), all N-Acyl homoserine lactones affected fecundity. The parent Compound (**1**) significantly affected egg laying down to 10 μM ($p < 0.0022$) when compared to DMSO. While both (*R*)-(compound **5**) and (*S*)- (compound **6**) enantiomers reduced egg production, the (*R*)- enantiomer was more effective. Of the compounds evaluated, Compound **3** was, once again, the most potent. Here, fecundity was significantly reduced at concentrations down to 2.5 μM ($p = 0.0413$) when compared to DMSO controls. While egg production was still affected at 1.25 μM, this was not statistically significant ($p > 0.9999$).

Next, we further evaluated the most potent analogue (compound **3**) on 3-week old juvenile worms and compared its effect to that induced by the racemic parent compound (**1**) (Fig 4).

For compound **1**, an EC_{50} of 4.3μM was noted with significant effect on worm motility down to 7.5 μM ($p = <0.0001$) (Fig 4A). For the thiolactone analogue (**3**), complete immobility was observed down to 0.94 μM ($p <0.0001$ for all concentrations) and an EC_{50} of 0.5 μM determined (Fig 4B). This data is consistent with the findings observed in schistosomula and the 7-week adult worm screens where incorporation of a thiolactone resulted in more effective anti-schistosomal activity. To additionally demonstrate that these N-Acyl homoserine lactones led to juvenile worm death, propidium iodide was utilised in compound **1** and **3** co-cultures (Fig 5).

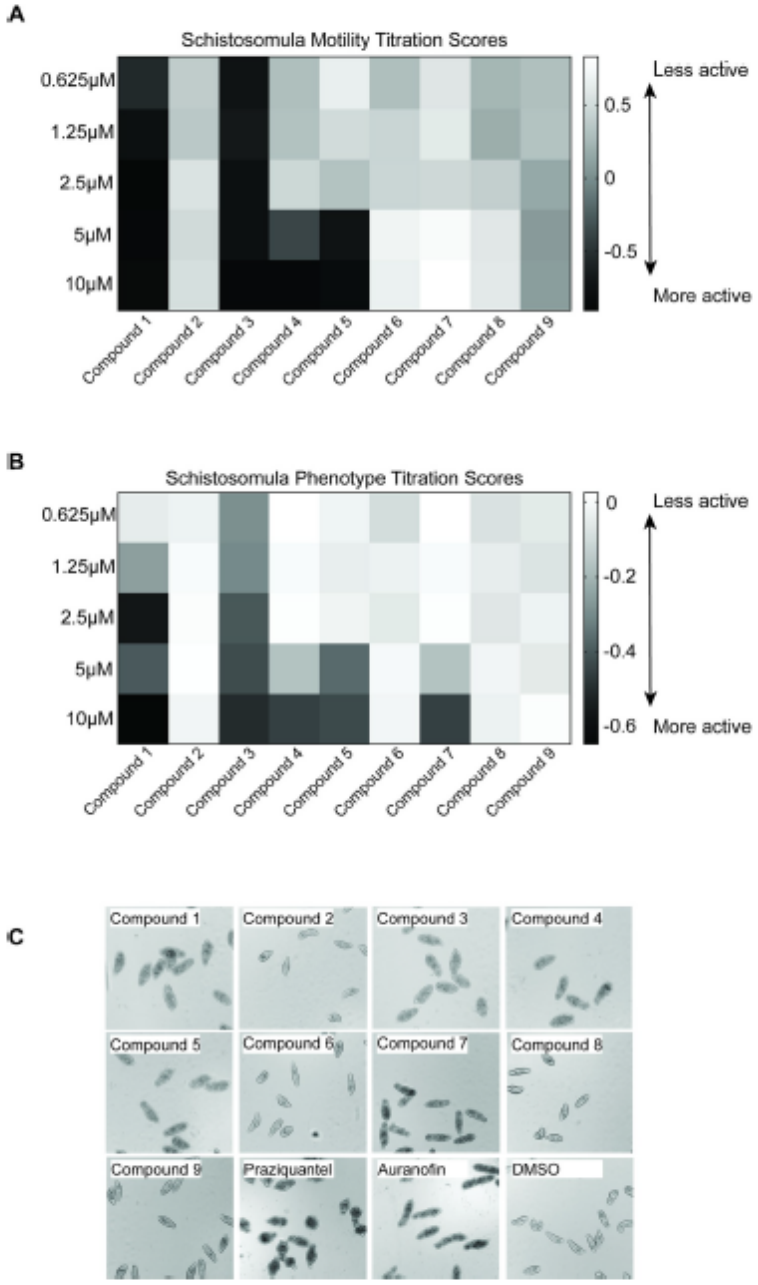

A
Schistosomula Motility Titration Scores
0.625μM
1.25μM
2.5μM
5μM
10μM
Compound 1
Compound 2
Compound 3
Compound 4
Compound 5
Compound 6
Compound 7
Compound 8
Compound 9
0.5
0
-0.5
Less active
More active
B
Schistosomula Phenotype Titration Scores
0.625μM
1.25μM
2.5μM
5μM
10μM
Compound 1
Compound 2
Compound 3
Compound 4
Compound 5
Compound 6
Compound 7
Compound 8
Compound 9
0
-0.2
-0.4
-0.6
Less active
More active
C
Compound 1
Compound 2
Compound 3
Compound 4
Compound 5
Compound 6
Compound 7
Compound 8
Compound 9
Praziquantel
Auranofin
DMSO

Fig 2. Anti-schistosomula activity of the eight analogues compared to parent compound 1. A total of 120 mechanically transformed schistosomula were co-cultured with each compound, titrated at doses between 10 and 0.625 μM. Test plates were incubated at 37˚C for 72 hrs in an atmosphere containing 5% CO_2. At 72hrs, schistosomula were scored using the Roboworm platform for both motility (**A**) and phenotype (**B**). Any compound that induced a score of below -0.35 for motility (**A**) and -0.15 for phenotype (**B**) were considered a hit. Black squares indicate the most positive effect on motility or phenotype; grey scale from dark grey to lighter shades of grey indicates a progressive reduced compound efficacy; white squares indicate no effect on either phenotype or motility. Z' values for this screen was 0.41741 for motility and 0.57275 for phenotype. (**C**) Phenotypes of schistosomula when treated with 10μM of each of the test compounds and controls.

https://doi.org/10.1371/journal.pntd.0008630.g002

The number of PI positive parasites when dosed with compound **1** was 100% at 15 and 7.5 μM concentrations, 13.3% at 3.75 μM and 0% for all other concentrations (Fig 5A). In contrast, co-culture in compound **3** resulted in 100% of the parasites being PI positive down to 0.94 μM (Fig 5B); this result tightly aligns to the motility scores quantified by light microscopy (Fig 4). Therefore, compounds **1** and **3** are not simply immobilising the juvenile parasites but are, in fact, killing them. To note, control juvenile parasites were also assessed for PI uptake (Fig 5C). For DMSO treated parasites, as expected with high motility scores, 0% of the parasites scored PI positive. Interestingly, PZQ treated parasites also displayed similar results to that of DMSO treated parasites where 0% of the juvenile worms were PI positive. This is in contradiction to PZQ-treated parasites scoring the lowest (0–1) for motility (Fig 4) and illustrates that, while this drug decreases motility, it does not kill juvenile stage parasites. Aurano-fin-treated parasites were all (100%) PI positive.

Evaluation of the indicative cytotoxic effect of these compounds against HepG2 cells was subsequently tested. Compounds **1–6** were titrated (from 200–1 μM) on HepG2 cells and co-cultivated for 24 hrs. A previous large scale mammalian cytotoxicity study indicated that maximal HepG2 cytotoxicity was observed within the first 24 hrs for 91% of the effective

Table 2. Calculated EC_{50}, CC_{50} and subsequent selectivity indices of N-acyl homoserines.

COMPOUND ID	SCHISTOSOMULA EC_{50}		ADULT WORM EC_{50}		JUVENILE WORM EC_{50}	HepG2 CC_{50}
	Phenotype	Motility	Male	Female		
1	5.6 CI (4.9–6.2)	3.8 CI (2.4–5.2)	8.2 CI (7.0–9.4)	8.3 CI **	4.3 CI **	43.5 CI (29.1–57.9)
2	>10 CI *	>10 CI *	> 20 CI *	>20 CI *		>200
3	2.3 CI (1.7–3.1)	1.9 CI (1.3–2.9)	4.2 CI (3.9–4.6)	3.9 CI (3.4–4.5)	0.5 CI **	18.7 CI (14.2–23.2)
4	4.9 CI (4.5–5.2)	4.6 CI (4.1–5.0)	>10 CI **	>10 CI **		70.8 CI (57–84.8)
5	4.0 CI **	3.1 CI **	>10 CI **	>10 CI **		50.6 CI (40.8–60.4)
6	>10 CI *	>10 CI *	16.0 CI (13.5–20.1)	>20 CI *		>100 CI*
SELECTIVITY INDICES						
1	7.9	11.5	5.3	5.2	10.1	
2						
3	8.1	9.8	4.5	4.8	37.4	
4	14.4	15.4				
5	12.7	16.3				
6			6.3			

Average EC_{50} calculated for each compound and life cycle stage of the parasite. Values are expressed as μM concentrations and 95% confidence intervals are shown. For compound 4 and 5, adult male EC_{50} is stated as >10 μM due to a rapid recovery in motility observed between 20 μM and 10 μM and, therefore, a more accurate estimate could not be obtained without further titration points between 20μM and 10μM.

* Confidence intervals (CI) were not calculated due to no compound effect seen at the highest dose tested

** Confidence intervals (CI) not calculable due to the motility scores obtained at the tested concentrations being too narrow (WHO-TDR scores of 3 and 4 only or 0 and 4 only), and therefore an accurate 95% CI could not be calculated.

https://doi.org/10.1371/journal.pntd.0008630.t002

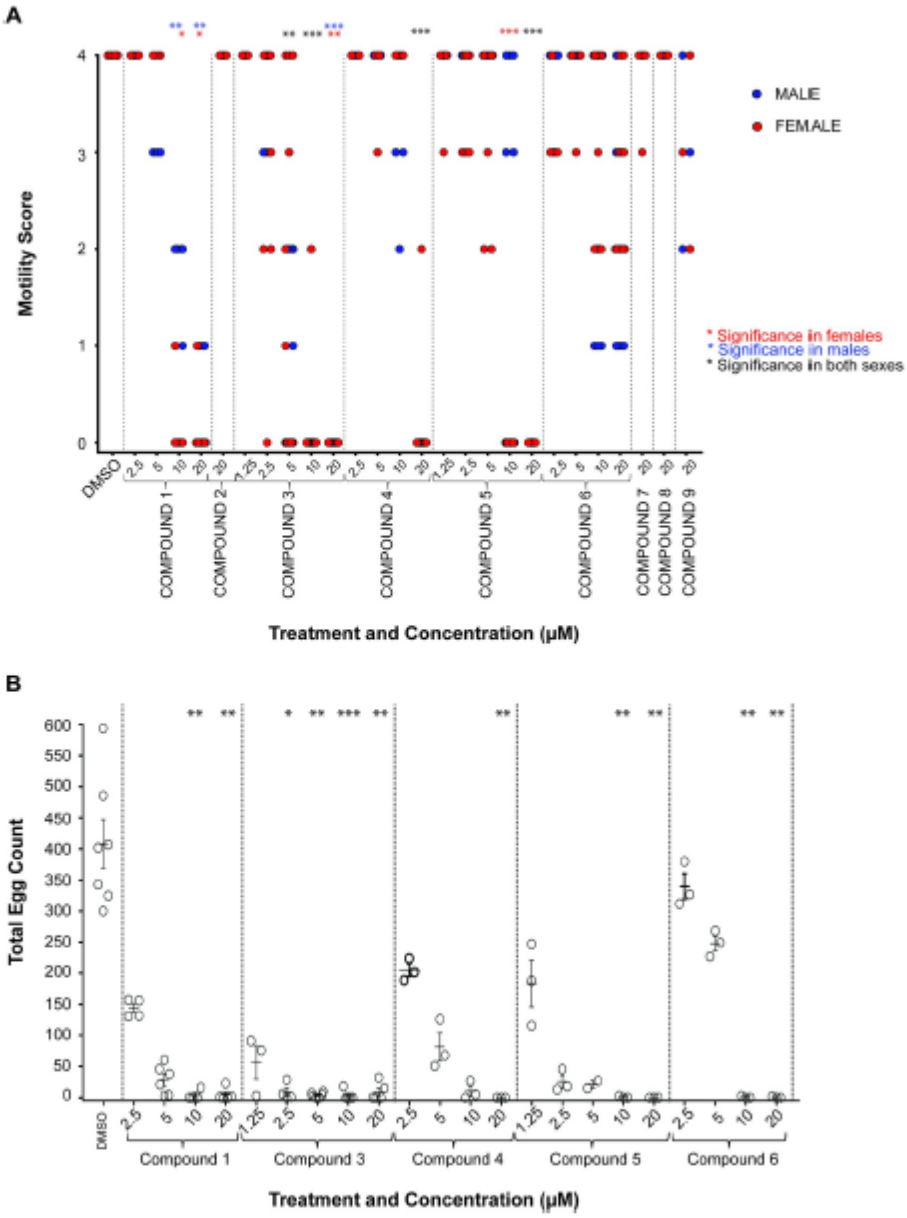


Fig 3. Adult schistosome motility and egg production are differentially affected by the nine N-acyl homoserine lactones. A) Adult *S. mansoni* worm pairs were cultured in decreasing compound concentrations of between 20 μM and 1.25 μM (not all concentrations were used for all compounds) for 72 hrs at 37°C, 5% CO_2. Parasite motility was evaluated for each sex and scored using the WHO-TDR scoring

system (0 = Dead parasite, 4 = Normal/Healthy movement). B) Culture media were collected from some adult co-cultures at 72 hrs and eggs present in the media were counted. *p* values are indicated as follows: * <0.05, ** <0.01, *** <0.001.

https://doi.org/10.1371/journal.pntd.0008630.g003

compounds [33]; therefore, 24 hrs continuous co-incubation of N-acyl homoserine lactones with HepG2 cells was selected for this study. The racemic compound (**1**) had an EC_{50} of 43.5 μM (Table 2) on HepG2 cells. Evaluation of the individual enantiomers demonstrated that (*R*)- and (*S*)- enantiomers had higher EC_{50} concentrations compared to the parent compound **1** (50.6 μM and >100 μM respectively). Incorporation of the thiolactone (**3**) resulted in greater cytotoxicity (EC_{50} = 18.7 μM) with a 2.3 fold increase in comparison to compound **1**.

With the EC_{50} data collected for schistosomula, adult and juvenile worms as well as the CC_{50} for HepG2, the selectivity indices (SI) could be determined for each of the compounds tested (Table 2). For all lifecycle stages tested, the thiolactone analogue (**3**) had the lowest EC_{50} values; however, it also had the poorest CC_{50} values of all the compounds tested, which resulted in some of the lowest SI scores except for juvenile worms where a SI of 37.4 was noted.

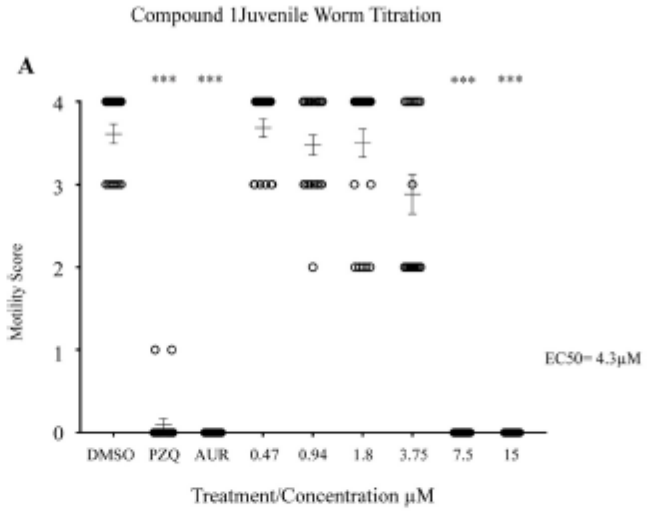
Due to limited information regarding potential targets of compound **1**, we next conducted an evaluation of structural similarities to previously published compounds as a first step towards this goal (Fig 6).

Using LeadMine and Datawarrior tools [30], compound **1** was found to be structurally similar to that of the N-butanoyl-l-homoserine lactones C4-HSL and PAI-2 (Fig 6A). Within *P. aeruginosa*, the N-3-oxo-dodecanoyl-l-homoserine lactone C12 and C4 signal through LasR and RhIR to facilitate quorum sensing [35] (Fig 6B). In addition to this function, both C4 and C12 HSLs regulate gene expression within *P. aeruginosa* as well as within several mammalian host cells [36–38]. Subsequently, we evaluated whether compound **1** may target similar *S. mansoni* orthologues to those used by *P. aeruginosa* in facilitating C4-HSL and C12-HSL signal transduction events. Upon BLATSP analyses of the *S. mansoni* genome, our findings failed to provide convincing evidence for LasR, LasI, RhIR, RHII (*P. aeruginosa)* orthologues. This suggests that our N-acyl homoserine analogue (**1**) is operating through differing mechanisms to that seen within bacteria, as has been postulated for mammalian systems [37, 38].

As Compound **1** was structurally similar to N-acylhomoserine lactones, a compound class involved in bacterial quorum sensing, we decided to assess its antimicrobial activity and determine if enantiomer separation or lactone ring substitution would change this activity. Compounds **1**, **3**, **5** and **6** were thus screened against representative Gram$^+$ (*Staphylococcus aureus*) and Gram$^-$ (*Escherichia coli*) species at final concentrations of 125, 62.5, 31.25, 15.6, 7.8 and 3.9 mg/L. Minimum inhibitory concentrations (MIC) were determined and represent the minimum concentration associated with no visible bacterial growth. The racemic compound (**1**) showed a MIC of 125 mg/L for both bacteria species, which was a similar value to that obtained for the (*R*)-enantiomer (**5**). In contrast, the (*S*)-enantiomer (**6**) did not show any activity (> 125 mg/L) on either bacterial species indicating that, similarly to the anthelmintic activity, the (*R*)-enantiomer alone is responsible for the antimicrobial activity (Table 3). When the lactone ring was replaced with a thiolactone substituent (**3**), the activity was considerably improved leading to a MIC of 31.25 mg/L for *S. aureus* (IC_{50} = 25.9 mg/L) and 62.5 mg/L for *E. coli* (IC_{50} = 52.7 mg/L) (Table 3).

Discussion

The drive to identify new anti-infectives is of paramount importance due to the continuous documentation and threat of drug resistant pathogens [39–42]. In particular, our research



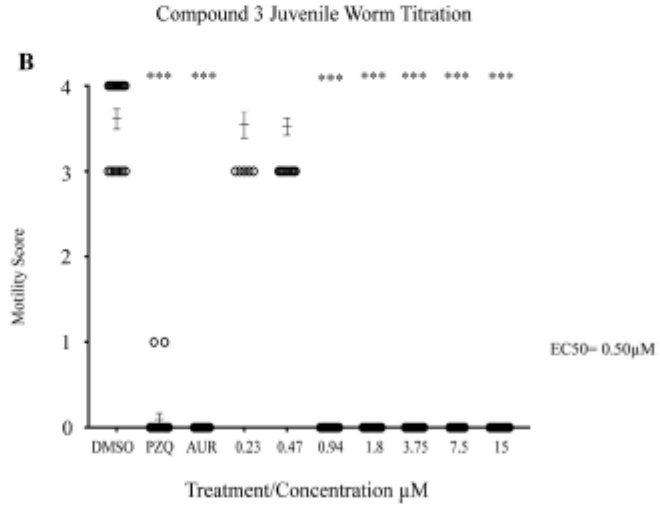


Fig 4. Three week juvenile worms are immobilised by N-Acyl homoserine lactones. Three-week old juvenile *S. mansoni* worms (n = 13–33 per well) were co-cultured with compounds (**1**) and (**3**) at concentrations spanning 15 μM —0.23 μM for 72 hrs at 37˚C in a humidified atmosphere containing 5% CO_2. Parasite motility was scored between 0–4 (0 = no movement/dead, 4 = full movement/healthy). DMSO negative controls were also included (1.25% final concentration) as well as two positive controls (15 μM PZQ and 15 μM auranofin; both in 1.25% DMSO). **A)** Effect of

compound (1) on juvenile worm motility. (B) Effect of compound (3) on juvenile worm motility. *p* values are indicated as follows: * <0.05, ** <0.01, *** <0.001.

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group has focused on identifying new chemotherapeutic compounds effective against *S. mansoni*, one of three major species responsible for human schistosomiasis.

In this study, 14 compounds were initially screened against schistosome parasites. It was evident that compound (**1**) had good activity against a range of lifecycle stages (schistosomula, adult and juvenile worms); therefore, a further 8 analogues (including the separated enantiomers) were generated. Interestingly the (*R*)-enantiomer was more effective when compared to the (*S*)-enantiomer in all of the parasitic assays conducted in this study; the (*R*)-enantiomer alone was also less toxic than parent compound **1** (EC_{50} 50.6μM and 43.5μM respectively). It is not surprising that different activities are associated with enantiomers; indeed PZQ itself is only effective via its (*R*)- enantiomer [43]. For example, Patterson *et al* demonstrated that the (*R*)-enantiomer of PA-824 (a promising antitubercular drug) had greater activity (when compared to the (*S*) enantiomer) against *L. donovani* during *in vivo* studies [44]. Furthermore, Paredes *et al* demonstrated that (*R*)-albendazole sulfoxide had greater anthelmintic activity against *T. solium* when compared to (*S*)-albendazole [45]. Our data, along with these and other parasitic studies [46], provides clear evidence that compound chirality represents an important consideration for drug discovery progression and putative target identification.

Upon further exploration of structural activity relationships, we found that elongation of the N-acyl chain led to decreased activity (**1** vs **2**; **3** vs **4**), while substitution of the lactone ring with a thiolactone improved activity (**1** vs **3**, **2** vs **4**) and led to the most effective compound **3**. It is reasonable to speculate that should **3** be available as the pure (*R*)-enantiomer, improvements in both anti-schistosomal potency and host cell cytotoxicity would be found (similar to those observed for compound **1** versus enantiomer pure (*R*) - **5** and (*S*) - **6**). A reduction in schistosome fecundity was noted in cultures co-incubated with **3** vs **4**; therefore, it could be argued that the addition of the methyl group in position 2 to the thiolactone analogue reduces parasite fecundity.

Comparative structural analysis revealed that compound **1**, having been previously characterised as an intermediary product of one-step procedures for producing $Gram^-$ N-acyl homoserines [47, 48], was similar to that of quorum sensing N-acyl homoserines. In recent years, quorum sensing has become a focus of development as a target for new anti-infective treatments [49]. Quorum sensing is a method by which both eukaryotic and prokaryotic cells regulate gene expression in response to fluctuations in cell population densities. Of relevance to parasites, *Trypanosoma brucei* is known to regulate its surrounding population by an analogous quorum sensing mechanism. In order to evade the host immunity response, trypanosomes adopt a slender morphology to proliferate, and in order to enter their transmission stage will transform into a stumpy form in which they stop proliferating [50]. This differentiation between two morphologically and molecular lifecycle stages is dependent on parasite density, and therefore can be seen as a type of quorum sensing [51, 52]. Much work has gone into identifying the mechanisms responsible for this density sensing to provide potential quorum sensing drug targets in trypanosomes [52]. Quorum sensing in schistosomes (or other metazoan endoparasites), to regulate lifecycle transitions, has not yet been identified.

With the identification of our parent compound having structural similarities to that of quorum-sensing N-acyl homoserines, we decided to evaluate compounds **1** and **3** (the most effective compound against *S. mansoni*), as well as the (*R*)- and (*S*)- enantiomers (**5** and **6** respectively) against both $Gram^+$ and $Gram^-$ bacteria to assess their potential as antimicrobials. Our findings in the bacteria screens were consistent with the *S. mansoni* data in that **3** was the

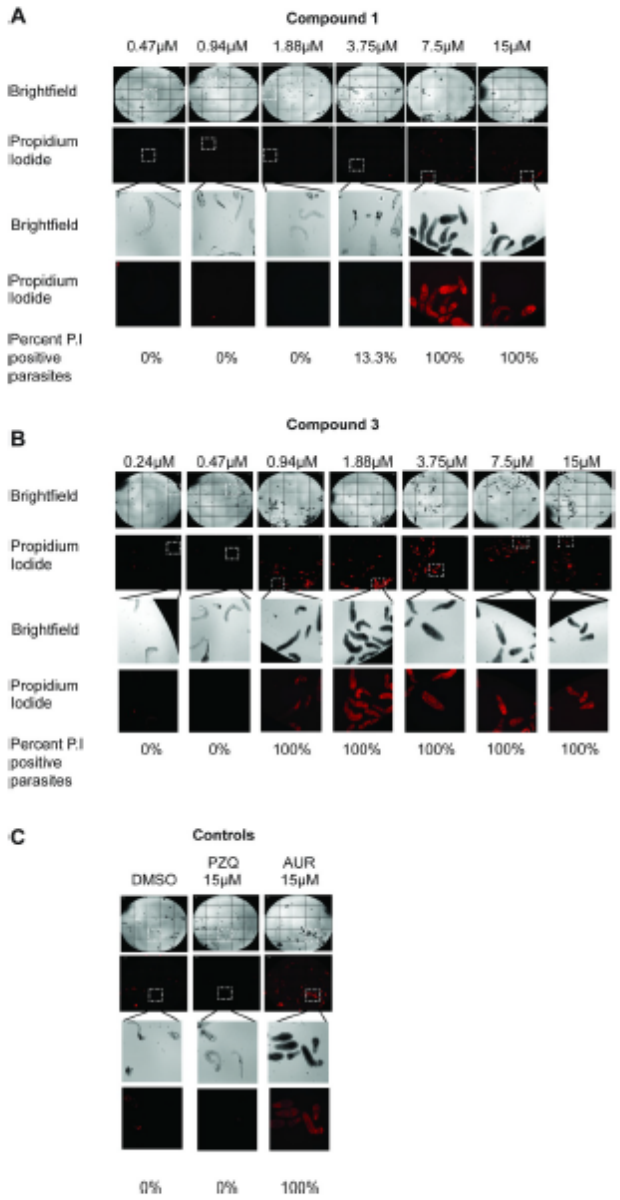

Fig 5. N-Acyl homoserine lactones kill juvenile stage schistosomes. Three week juvenile schistosomes, incubated with compounds (1), (3) or controls for 72 hrs, were subsequently cultured with PI at a final concentration of 2 μg/mL for 15 minutes at 37 °C in an environment containing 5% CO_2. PI positive parasites (dead) were counted and the percent live vs dead in each well is indicated. A) Quantification of compound (1) mediated juvenile death. B)

Quantification of compound (3) mediated juvenile death. C) Quantification of juvenile deaths in co-cultures containing 15 μM PZQ (in 1.25% DMSO), 15 μM auranofin (in 1.25% DMSO) and 1.25% DMSO.

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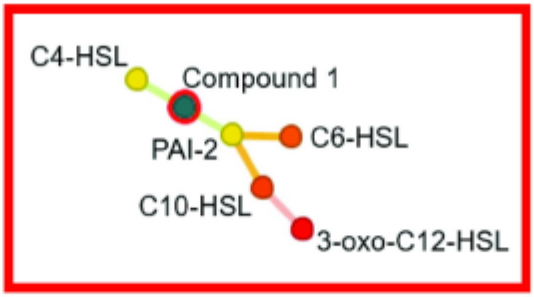
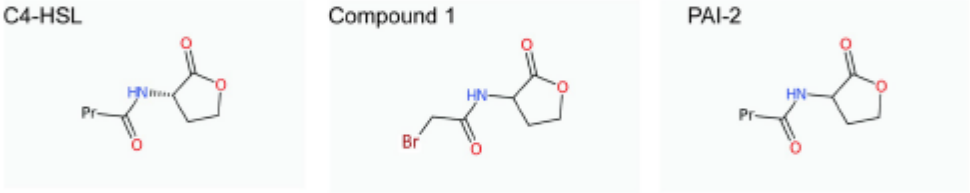
most potent, although only moderately active in comparison to clinical standards [53], N-acyl homoserine against Gram$^+$ bacteria and that the (*R*)- (**5**) was more effective than the (*S*)- (**6**) enantiomer. Furthermore, the thiolactone (**3**) had a 4- and 2- fold improvement in activity against *S. aureus* and *E. coli* respectively, compared to compound **1**. It is interesting that N-acyl homoserines should have greater effect against a Gram$^+$ bacteria, which in natural environments do not utilise these compounds as part of their quorum sensing regulation [54]. An explanation for increased inhibition of *S. aureus* growth when treated with compound **3** may be due to cross-inhibition between bacteria species. It has previously been demonstrated that bacteria undergo strategies to reduce the efficacy of quorum sensing used by competitor bacterial species and thus increase the likelihood of their own survival [54, 55]. The role of N-acyl homoserines in this process of cell to cell bacterial communication has been clearly demonstrated for *S. aureus* and *P. aeruginosa* [56]. Here, N-acyl homoserines (e.g. 3-oxo-C_{12}-HSL) produced by Gram$^-$ *P. aeruginosa* inhibit growth and virulence factor production of Gram$^+$ *S. aureus*. When co-inhabiting infected tissues, this N-acyl homoserine-mediated strategy of cell communication provides *P. aeruginosa* with a competitive advantage over *S. aureus*. Other studies have also demonstrated a role for N-acyl homoserines in mediating pathogen population densities; for example, *Candida albicans* filamentation can be suppressed by 3-oxo-C_{12}-HSL (homoserine lactone) produced by *P. aeruginosa* [57]. It is, therefore, likely that our synthetic N-acyl homoserine analogues (especially thiolactone containing compound **3**) inhibit *S. aureus* growth in a similar manner as the above examples or those additional ones found in the literature illustrate (54, 55). For example, McInnis *et al* have previously designed and validated a library of 21 thiolactone analogues that were either naturally occurring or non-native N-acyl homoserines and evaluated their effect as agonists and antagonists of LuxR-type quorum sensing receptors in *P.aeruginosa* (LasR), *V. fischeri* (LuxR), and *A.tumefaciens* (TraR). They were able to demonstrate the improved potency of the thiolactone analogues in comparison to the N-acyl homoserine parent compound (similar to our findings here) [58].

In *P. aeruginosa*, its N-acyl homoserines primarily function through the transcription factors LasR and RhlR, but similar orthologues were not identified in the *S. mansoni* genome. However, it has also been shown that bacterial N-acyl homoserines mediate cross-Kingdom communication by directly interacting with membrane lipids; this mechanism of action may be responsible for the anti-schistosomal effects observed in our studies. For example, long chain N-acyl homoserines (e.g. 3-oxo-C_{12}-HSL) can insert into cholesterol-containing microdomains leading to changes in dipole potential, integral protein re-organisation and subsequent activation of signal transduction cascades [36, 59]. The intra-mammalian schistosome is covered by two tightly opposed lipid bilayers including an inner plasma membrane and an outer membranocalyx [60–62]. The membranocalyx has the highest density of intramembranous particles [63] comprised of nutrient transporters, transmembrane proteins and other gene products of currently unknown function [64–66]. Also, it is rich in neutral lipids, cholesterol and phospholipids [67–70]. It is possible that the compounds analysed in this study may bind to the cholesterol rich regions within this membranocalyx layer, which subsequently causes conformational changes to the transmembrane proteins affecting their downstream signalling cascades. This could ultimately result in the phenotypes observed in our studies. However, as compounds displaying greater lipophilicity did not necessarily increase anthelmintic activity, features such as chemical space/composition and size may be more important. Further experimentation is required to tease apart the mode of action of these anthelmintic compounds.

A


C4-HSL
Compound 1
PAI-2
C6-HSL
C10-HSL
3-oxo-C12-HSL


C4-HSL
Compound 1
PAI-2

B

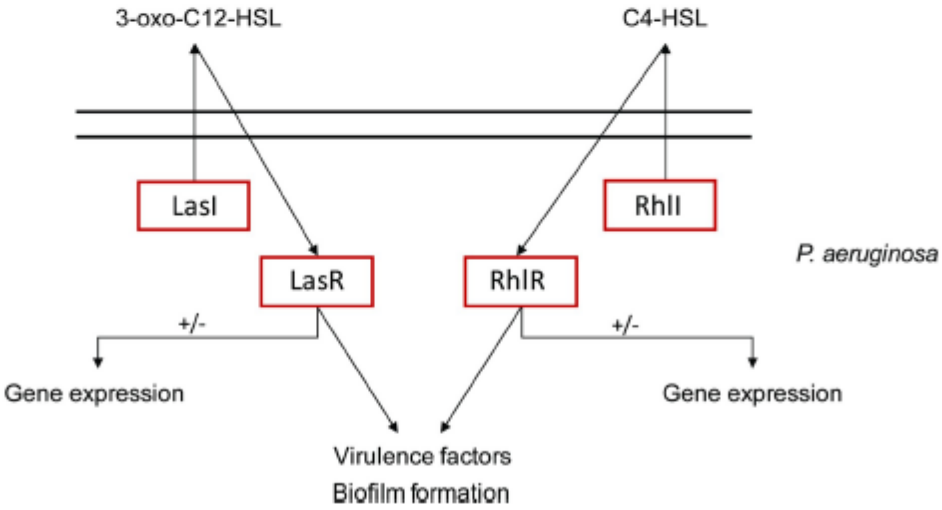

3-oxo-C12-HSL
C4-HSL
LasI
RhlI
P. aeruginosa
LasR
RhlR
+/-
+/-
Gene expression
Gene expression
Virulence factors
Biofilm formation

Fig 6. *In silico* approach to identify potential targets of compound 1 within *S. mansoni*. A) FragFP output in DataWarrior demonstrates that Compound 1 is structurally related to C4-HSL (formerly called PAI-2 [34]); C4-HSL is an N-acyl homoserines involved in quorum sensing within *P. aeruginosa*. Substitution of the methyl and bromine groups (found in compound 1) is observed with propyl and stereochemistry modifications. B) Pathways in *P. aeruginosa* that utilise both C4- and C12- HSLs (LasI = acyl-homoserine-lactone synthase; LasR = transcriptional activator; RhlR = regulatory protein RhlI = acyl-homoserine-lactone synthase). Highlighted (red rectangles) are those *P. aeruginosa* proteins used in BLASTp analysis of the *S. mansoni* genome (v7.0).

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Table 3. Antibacterial activity (MIC) of the tested compounds and antibacterial selectivity of compound 3 (IC_{50}).

Compound ID	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	CC_{50} (μM) (HepG2)	Selectivity Index *S. aureus* / *E.coli*
	MIC		IC_{50} (μM)			
1	**125** [563.1]	**125** [563.1]				
3	**31.3** [131.2]	**62.5** [262.5]	**25.9** (23.3–30.7)	**52.7** (42.3–66.9)	**18.7** (14.2–23.2)	0.72 / 0.35
5	**125** [563.1]	**125** [563.1]				
6	**> 125** [> 563.1]	**> 125** [> 563.1]				

Minimum inhibitory concentration of compounds expressed as mg/L [μM] against *Staphylococcus aureus* and *Escherichia coli*. IC_{50} against *S. aureus* and *E. coli* determined from three independent experiments and expressed as mg/L [μM] with 95% confidence interval (in parentheses). Selectivity indices were calculated from HepG2 CC_{50} values.

https://doi.org/10.1371/journal.pntd.0008630.t003

To the best of our knowledge, this is the first study to demonstrate that synthetic bacterial N-acyl homoserines analogues are effective against *S. mansoni*. This is a new avenue of investigation as these compounds represent novel starting points for future anthelmintic drug discovery studies. We have also demonstrated the importance of enantiomer contribution, in line with previous findings, and that some molecules display cross-pathogen activity. When taken together, these findings could inform the development of new, broad-acting anti-infectives.

Supporting information

S1 Protocol. Detailed materials and methods for chemical synthesis of compounds 1–9.
(DOCX)

S1 Table. Structural details of all compounds screened against *S. mansoni* in this study. The first 14 compounds represent the initial compounds screened. The following 8 are compound 1 analogues.
(XLSX)

S1 Fig. Initial schistosomula screens of the 14 compounds and their intermediate analogues. A total of 120 mechanically transformed schistosomula were co-cultured with each compound, titrated at doses between 10 and 0.625 μM. Test plates were incubated at 37˚C for 72 hrs in an atmosphere containing 5% CO_2. At 72hrs, schistosomula were scored using the Roboworm platform for both motility and phenotype as previously described [17, 25]. A) Of the 14 compounds evaluated, two fell within the hit region. Z' scores for motility and phenotype were 0.40079 and 0.56846 respectively. B) Further titration of the two hit compounds resulted in good compound effect being observed for compound 1 down to a concentration of 1.25 μM. Z' scores for motility and phenotype were 0.37864 and 0.47882 respectively.
*Average EC_{50} value across motility and phenotype is presented for each compound.
(TIF)

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Methodology: Helen Whiteland, Alessandra Crusco.

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Resources: Josephine Forde-Thomas, Patrick J. Murphy.

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Writing – review & editing: Alessandra Crusco, Lisa W. Bloemberg, Jamie Tibble-Howlings, Josephine Forde-Thomas, Avril Coghlan, Patrick J. Murphy, Karl F. Hoffmann.

References

1. Organization WH. Working to overcome the global impact of neglected tropical diseases: first WHO report on neglected tropical diseases. Geneva: World Health Organization; 2010.
2. Hotez PJ, Alvarado M, Basáñez M-G, Bolliger I, Bourne R, Boussinesq M, et al. The global burden of disease study 2010: interpretation and implications for the neglected tropical diseases. PLoS neglected tropical diseases. 2014; 8(7):e2865. https://doi.org/10.1371/journal.pntd.0002865 PMID: 25058013
3. Frieri M, Kumar K, Boutin A. Antibiotic resistance. Journal of infection and public health. 2017; 10 (4):369–78. https://doi.org/10.1016/j.jiph.2016.08.007 PMID: 27616769
4. Morschhäuser J. Regulation of multidrug resistance in pathogenic fungi. Fungal Genetics and Biology. 2010; 47(2):94–106. https://doi.org/10.1016/j.fgb.2009.08.002 PMID: 19665571
5. Chevalier FD, Le Clec'h W, McDew-White M, Menon V, Guzman MA, Holloway SP, et al. Oxamniquine resistance alleles are widespread in Old World Schistosoma mansoni and predate drug deployment. PLoS pathogens. 2019; 15(10).
6. Patrick J, Anderson F, Wilson K, McCormick I, Skuce P, O'Rourke J. Triclabendazole-resistant liver fluke: issues and strategies. Livestock. 2018; 23(Sup5):4–14.
7. Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM. Evidence of artemisinin-resistant malaria in western Cambodia. New England Journal of Medicine. 2008; 359(24):2619–20. https://doi.org/10.1056/NEJMc0805011 PMID: 19064625
8. Lira R, Sundar S, Makharia A, Kenney R, Gam A, Saraiva E, et al. Evidence that the high incidence of treatment failures in Indian kala-azar is due to the emergence of antimony-resistant strains of Leishmania donovani. The Journal of infectious diseases. 1999; 180(2):564–7. https://doi.org/10.1086/314896 PMID: 10395884
9. Shlaes DM, Projan SJ, Edwards J. Antibiotic discovery: state of the state. ASM News-American Society for Microbiology. 2004; 70(6):275–81.
10. Bush K. Why it is important to continue antibacterial drug discovery. ASM News-American Society for Microbiology. 2004; 70(6):282–7.

11. Projan SJ. Why is big Pharma getting out of antibacterial drug discovery? Current opinion in microbiology. 2003; 6(5):427–30. https://doi.org/10.1016/j.mib.2003.08.003 PMID: 14572532

12. El Ridi RA, Tallima HA-M. Novel therapeutic and prevention approaches for schistosomiasis. Journal of Advanced Research. 2013; 4(5):467–78. https://doi.org/10.1016/j.jare.2012.05.002 PMID: 25685454

13. Colley D, Secor W. Immunology of human schistosomiasis. Parasite immunology. 2014; 36(8):347–57. https://doi.org/10.1111/pim.12087 PMID: 25142505

14. Vale N, Gouveia MJ, Rinaldi G, Brindley PJ, Gärtner F, da Costa JMC. Praziquantel for schistosomiasis: single-drug metabolism revisited, mode of action, and resistance. Antimicrobial agents and chemotherapy. 2017; 61(5):e02582–16. https://doi.org/10.1128/AAC.02582-16 PMID: 28264841

15. Cioli D, Pica-Mattoccia L, Basso A, Guidi A. Schistosomiasis control: praziquantel forever? Molecular and biochemical parasitology. 2014; 195(1):23–9. https://doi.org/10.1016/j.molbiopara.2014.06.002 PMID: 24955523

16. Edwards J, Brown M, Peak E, Bartholomew B, Nash RJ, Hoffmann KF. The diterpenoid 7-keto-sempervirol, derived from Lycium chinense, displays anthelmintic activity against both Schistosoma mansoni and Fasciola hepatica. PLoS neglected tropical diseases. 2015; 9(3):e0003604. https://doi.org/10.1371/journal.pntd.0003604 PMID: 25768432

17. Crusco A, Bordoni C, Chakroborty A, Whatley KC, Whiteland H, Westwell AD, et al. Design, synthesis and anthelmintic activity of 7-keto-sempervirol analogues. European journal of medicinal chemistry. 2018; 152:87–100. https://doi.org/10.1016/j.ejmech.2018.04.032 PMID: 29698860

18. Whiteland HL, Chakroborty A, Forde-Thomas JE, Crusco A, Cookson A, Hollinshead J, et al. An Abies procera-derived tetracyclic diterpene containing a steroid-like nucleus core and a lactone side chain attenuates in vitro survival of both Fasciola hepatica and Schistosoma mansoni. International Journal for Parasitology: Drugs and Drug Resistance. 2018; 8(3):465–74. https://doi.org/10.1016/j.ijpddr.2018.10.009 PMID: 30399512

19. Whatley K, Padalino G, Whiteland H, Geyer K, Hulme B, Chalmers I, et al. The repositioning of epigenetic probes/inhibitors identifies new anti-schistosomal lead compounds and chemotherapeutic targets. BioRxiv. 2019:729814.

20. Padalino G, Ferla S, Brancale A, Chalmers IW, Hoffmann KF. Combining bioinformatics, cheminformatics, functional genomics and whole organism approaches for identifying epigenetic drug targets in Schistosoma mansoni. International Journal for Parasitology: Drugs and Drug Resistance. 2018; 8(3):559–70. https://doi.org/10.1016/j.ijpddr.2018.10.005 PMID: 30455056

21. Crusco A, Baptista R, Bhowmick S, Beckmann M, Mur LA, Westwell AD, et al. The anti-mycobacterial activity of a diterpenoid-like molecule operates through nitrogen and amino acid starvation. Frontiers in Microbiology. 2019; 10:1444. https://doi.org/10.3389/fmicb.2019.01444 PMID: 31293560

22. Williams P. Quorum sensing, communication and cross-kingdom signalling in the bacterial world. Microbiology. 2007; 153(12):3923–38.

23. Bickle Q, Doenhoff M. Comparison of the live vaccine potential of different geographic isolates of Schistosoma mansoni. Journal of helminthology. 1987; 61(3):191–5. https://doi.org/10.1017/s0022149x00010002 PMID: 3117874

24. Colley DG, Wikel SK. Schistosoma mansoni: simplified method for the production of schistosomules. Exp Parasitol. 1974; 35(1):44–51. https://doi.org/10.1016/0014-4894(74)90005-8 PMID: 4815018

25. Ramirez B, Bickle Q, Yousif F, Fakorede F, Mouries MA, Nwaka S. Schistosomes: challenges in compound screening. Expert Opin Drug Discov. 2007; 2(s1):S53–61. https://doi.org/10.1517/17460441.2.S1.S53 PMID: 23489033

26. Peak E, Chalmers IW, Hoffmann KF. Development and validation of a quantitative, high-throughput, fluorescent-based bioassay to detect schistosoma viability. PLoS neglected tropical diseases. 2010; 4(7):e759. https://doi.org/10.1371/journal.pntd.0000759 PMID: 20668553

27. Nur EAM, Yousaf M, Ahmed S, Al-Sheddi ES, Parveen I, Fazakerley DM, et al. Neoclerodane Diterpenoids from Reehal Fatima, Teucrium yemense. J Nat Prod. 2017; 80(6):1900–8. https://doi.org/10.1021/acs.jnatprod.7b00188 PMID: 28581290

28. Microbiology ECfASTotESoC, Diseases I. Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. Clinical Microbiology and Infection. 2003; 9(8):ix–xv.

29. Lowe DM, Sayle RA. LeadMine: a grammar and dictionary driven approach to entity recognition. Journal of cheminformatics. 2015; 7(1):S5.

30. Sander T, Freyss J, von Korff M, Rufener C. DataWarrior: an open-source program for chemistry aware data visualization and analysis. Journal of chemical information and modeling. 2015; 55(2):460–73. https://doi.org/10.1021/ci500588j PMID: 25558886

31. Howe KL, Bolt BJ, Cain S, Chan J, Chen WJ, Davis P, et al. WormBase 2016: expanding to enable helminth genomic research. Nucleic acids research. 2016; 44(D1):D774–D80. https://doi.org/10.1093/nar/gkv1217 PMID: 26578572

32. Gray DJ, Ross AG, Li Y-S, McManus DP. Diagnosis and management of schistosomiasis. Bmj. 2011; 342:d2651. https://doi.org/10.1136/bmj.d2651 PMID: 21586478

33. Hsieh J-H, Huang R, Lin J-A, Sedykh A, Zhao J, Tice RR, et al. Real-time cell toxicity profiling of Tox21 10K compounds reveals cytotoxicity dependent toxicity pathway linkage. PloS one. 2017; 12(5): e0177902. https://doi.org/10.1371/journal.pone.0177902 PMID: 28531190

34. Pearson JP, Van Delden C, Iglewski BH. Active efflux and diffusion are involved in transport of Pseudomonas aeruginosa cell-to-cell signals. Journal of bacteriology. 1999; 181(4):1203–10. https://doi.org/10.1128/JB.181.4.1203-1210.1999 PMID: 9973347

35. Pesci EC, Pearson JP, Seed PC, Iglewski BH. Regulation of las and rhl quorum sensing in Pseudomonas aeruginosa. Journal of bacteriology. 1997; 179(10):3127–32. https://doi.org/10.1128/jb.179.10.3127-3132.1997 PMID: 9150205

36. Davis BM, Jensen R, Williams P, O'Shea P. The interaction of N-acylhomoserine lactone quorum sensing signaling molecules with biological membranes: implications for inter-kingdom signaling. PloS one. 2010; 5(10):e13522. https://doi.org/10.1371/journal.pone.0013522 PMID: 20975958

37. Kravchenko VV, Kaufmann GF, Mathison JC, Scott DA, Katz AZ, Wood MR, et al. N-(3-oxo-acyl) homoserine lactones signal cell activation through a mechanism distinct from the canonical pathogen-associated molecular pattern recognition receptor pathways. Journal of Biological Chemistry. 2006; 281 (39):28822–30. https://doi.org/10.1074/jbc.M606613200 PMID: 16893899

38. Hooi DS, Bycroft BW, Chhabra SR, Williams P, Pritchard DI. Differential immune modulatory activity of Pseudomonas aeruginosa quorum-sensing signal molecules. Infection and immunity. 2004; 72 (11):6463–70. https://doi.org/10.1128/IAI.72.11.6463-6470.2004 PMID: 15501777

39. Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, et al. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. The Lancet Infectious Diseases. 2018; 18(3):318–27. https://doi.org/10.1016/S1473-3099(17)30753-3 PMID: 29276051

40. Nikaido H. Multidrug resistance in bacteria. Annual review of biochemistry. 2009; 78:119–46. https://doi.org/10.1146/annurev.biochem.78.082907.145923 PMID: 19231985

41. Donia M, Hamann MT. Marine natural products and their potential applications as anti-infective agents. The Lancet infectious diseases. 2003; 3(6):338–48. https://doi.org/10.1016/s1473-3099(03)00655-8 PMID: 12781505

42. Doenhoff MJ, Kusel JR, Coles GC, Cioli D. Resistance of Schistosoma mansoni to praziquantel: is there a problem? Transactions of the Royal Society of Tropical Medicine and Hygiene. 2002; 96 (5):465–9. https://doi.org/10.1016/s0035-9203(02)90405-0 PMID: 12474468

43. Meister I, Ingram-Sieber K, Cowan N, Todd M, Robertson MN, Keiser J, et al. Activity of praziquantel enantiomers and main metabolites against Schistosoma mansoni. Antimicrobial agents and chemotherapy. 2014; 58(9):5466–72. https://doi.org/10.1128/AAC.02741-14 PMID: 24982093

44. Patterson S, Wyllie S, Stojanovski L, Perry MR, Simeons FR, Norval S, et al. The R enantiomer of the antitubercular drug PA-824 as a potential oral treatment for visceral leishmaniasis. Antimicrobial agents and chemotherapy. 2013; 57(10):4699–706. https://doi.org/10.1128/AAC.00722-13 PMID: 23856774

45. Paredes A, de Campos Lourenço T, Marzal M, Rivera A, Dorny P, Mahanty S, et al. In vitro analysis of albendazole sulfoxide enantiomers shows that (+)-(R)-albendazole sulfoxide is the active enantiomer against Taenia solium. Antimicrobial agents and chemotherapy. 2013; 57(2):944–9. https://doi.org/10.1128/AAC.01465-12 PMID: 23229490

46. Nanayakkara ND, Ager AL, Bartlett MS, Yardley V, Croft SL, Khan IA, et al. Antiparasitic activities and toxicities of individual enantiomers of the 8-aminoquinoline 8-[(4-amino-1-methylbutyl) amino]-6-methoxy-4-methyl-5-[3, 4-dichlorophenoxy] quinoline succinate. Antimicrobial agents and chemotherapy. 2008; 52(6):2130–7. https://doi.org/10.1128/AAC.00645-07 PMID: 18378716

47. Persson T, Hansen TH, Rasmussen TB, Skindersø ME, Givskov M, Nielsen J. Rational design and synthesis of new quorum-sensing inhibitors derived from acylated homoserine lactones and natural products from garlic. Organic & biomolecular chemistry. 2005; 3(2):253–62.

48. Stacy DM, Le Quement ST, Hansen CL, Clausen JW, Tolker-Nielsen T, Brummond JW, et al. Synthesis and biological evaluation of triazole-containing N-acyl homoserine lactones as quorum sensing modulators. Organic & biomolecular chemistry. 2013; 11(6):938–54.

49. Kaufmann GF, Park J, Janda KD. Bacterial quorum sensing: a new target for anti-infective immunotherapy. Expert opinion on biological therapy. 2008; 8(6):719–24. https://doi.org/10.1517/14712598.8.6.719 PMID: 18476783

50. Vickerman K. Developmental cycles and biology of pathogenic trypanosomes. British medical bulletin. 1985; 41(2):105–14. https://doi.org/10.1093/oxfordjournals.bmb.a072036 PMID: 3928017

51. Vassella E, Reuner B, Yutzy B, Boshart M. Differentiation of African trypanosomes is controlled by a density sensing mechanism which signals cell cycle arrest via the cAMP pathway. Journal of cell science. 1997; 110(21):2661–71.

52. Mony BM, MacGregor P, Ivens A, Rojas F, Cowton A, Young J, et al. Genome-wide dissection of the quorum sensing signalling pathway in Trypanosoma brucei. Nature. 2014; 505(7485):681. https://doi.org/10.1038/nature12864 PMID: 24336212

53. Testing TECoAS. Routine and extended internal quality control for MIC determination and disk diffusion as recommended by EUCAST 2020 [Version 10.0]Available from: http://www.eucast.org.

54. Bassler BL. Small talk: cell-to-cell communication in bacteria. Cell. 2002; 109(4):421–4. https://doi.org/10.1016/s0092-8674(02)00749-3 PMID: 12086599

55. Taga ME, Bassler BL. Chemical communication among bacteria. Proceedings of the National Academy of Sciences. 2003; 100(suppl 2):14549–54.

56. Qazi S, Middleton B, Muharram SH, Cockayne A, Hill P, O'Shea P, et al. N-acylhomoserine lactones antagonize virulence gene expression and quorum sensing in Staphylococcus aureus. Infection and immunity. 2006; 74(2):910–9. https://doi.org/10.1128/IAI.74.2.910-919.2006 PMID: 16428734

57. Hogan DA, Vik Å, Kolter R. A Pseudomonas aeruginosa quorum-sensing molecule influences Candida albicans morphology. Molecular microbiology. 2004; 54(5):1212–23. https://doi.org/10.1111/j.1365-2958.2004.04349.x PMID: 15554963

58. McInnis CE, Blackwell HE. Thiolactone modulators of quorum sensing revealed through library design and screening. Bioorganic & medicinal chemistry. 2011; 19(16):4820–8.

59. Song D, Meng J, Cheng J, Fan Z, Chen P, Ruan H, et al. Pseudomonas aeruginosa quorum-sensing metabolite induces host immune cell death through cell surface lipid domain dissolution. Nature microbiology. 2019; 4(1):97–111. https://doi.org/10.1038/s41564-018-0290-8 PMID: 30510173

60. Hockley DJ, McLaren DJ. Schistosoma mansoni: changes in the outer membrane of the tegument during development from cercaria to adult worm. International journal for parasitology. 1973; 3(1):13–20. https://doi.org/10.1016/0020-7519(73)90004-0 PMID: 4687430

61. McLaren DJ, HOCKLEY DJ. Blood flukes have a double outer membrane. Nature. 1977; 269 (5624):147–9. https://doi.org/10.1038/269147a0 PMID: 71658

62. Skelly PJ, Wilson RA. Making sense of the schistosome surface. Advances in parasitology. 2006; 63:185–284. https://doi.org/10.1016/S0065-308X(06)63003-0 PMID: 17134654

63. Hockley D, McLaren DJ, Ward BJ, Nermut M. A freeze-fracture study of the tegumental membrane of Schistosoma mansoni (Platyhelminthes: Trematoda). Tissue and Cell. 1975; 7(3):485–96. https://doi.org/10.1016/0040-8166(75)90020-8 PMID: 1179410

64. van Balkom BW, van Gestel RA, Brouwers JF, Krijgsveld J, Tielens AG, Heck AJ, et al. Mass Spectrometric Analysis of the Schistosoma mansoni Tegumental Sub-proteome. Journal of proteome research. 2005; 4(3):958–66. https://doi.org/10.1021/pr050036w PMID: 15952743

65. Braschi S, Borges WC, Wilson RA. Proteomic analysis of the shistosome tegument and its surface membranes. Memorias do Instituto Oswaldo Cruz. 2006; 101:205–12. https://doi.org/10.1590/s0074-02762006000900032 PMID: 17308771

66. Castro-Borges W, Simpson DM, Dowle A, Curwen RS, Thomas-Oates J, Beynon RJ, et al. Abundance of tegument surface proteins in the human blood fluke Schistosoma mansoni determined by QconCAT proteomics. Journal of Proteomics. 2011; 74(9):1519–33. https://doi.org/10.1016/j.jprot.2011.06.011 PMID: 21704203

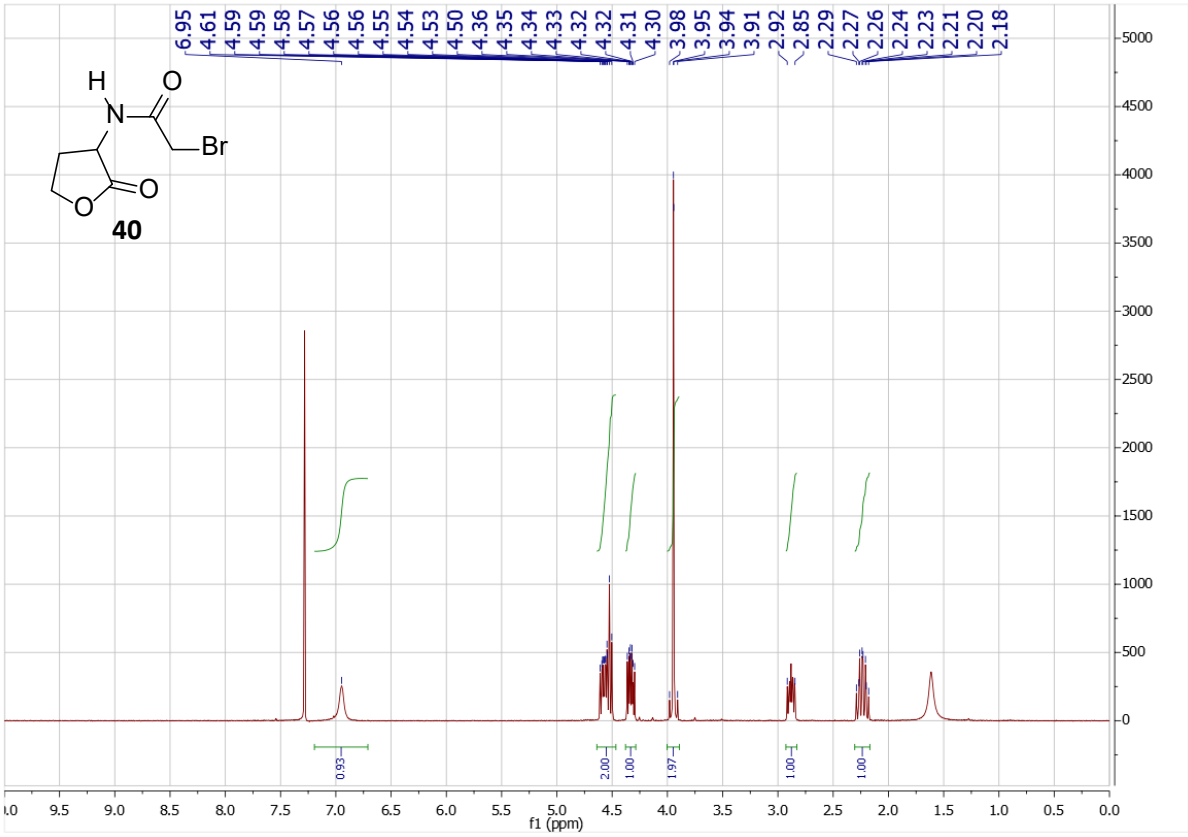
67. Retta K, deWalick S, Schmitz M, Yazdanbakhsh M, Tielens AG, Brouwers JF, et al. The tegumental surface membranes of Schistosoma mansoni are enriched in parasite-specific phospholipid species. International journal for parasitology. 2015; 45(9–10):629–36. https://doi.org/10.1016/j.ijpara.2015.03.011 PMID: 25975668

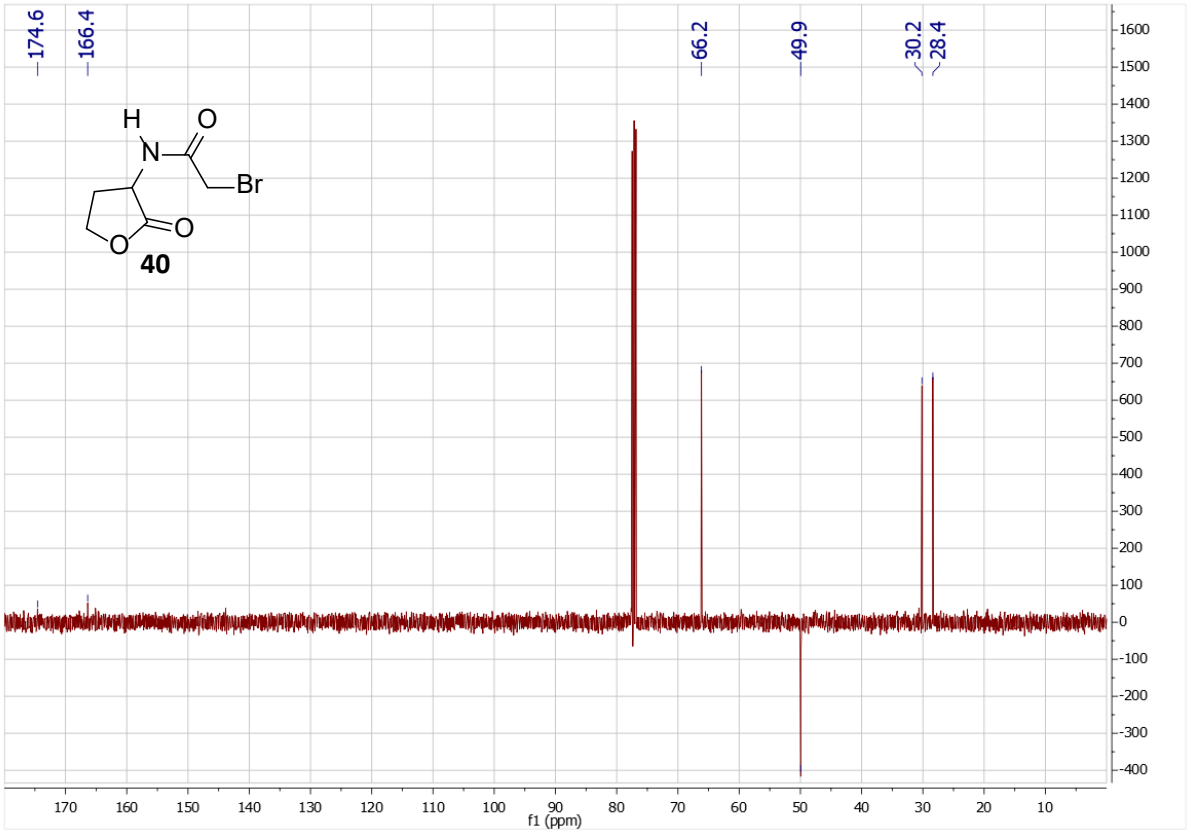
68. Tallima H, Hamada M, El Ridi R. Evaluation of cholesterol content and impact on antigen exposure in the outer lipid bilayer of adult schistosomes. Parasitology. 2007; 134(12):1775–83.

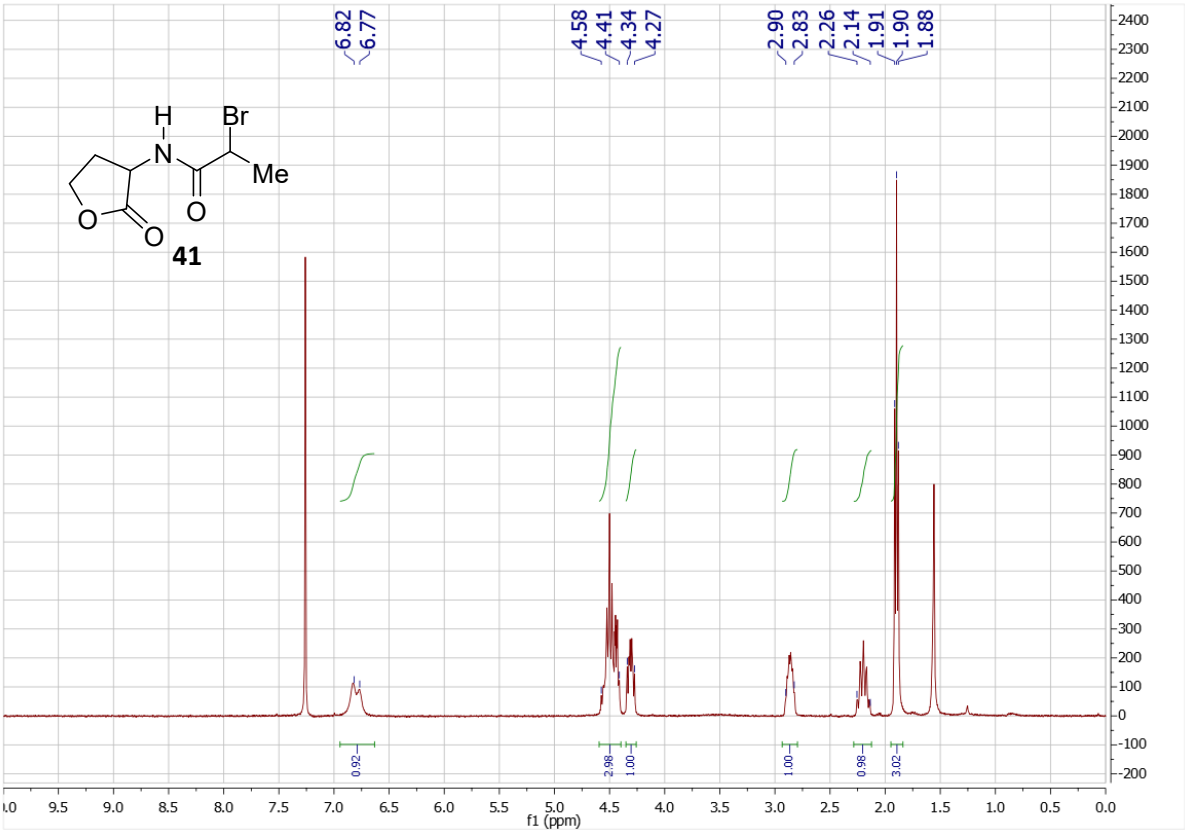
69. Rogers MV, McLaren DJ. Analysis of total and surface membrane lipids of Schistosoma mansoni. Molecular and biochemical parasitology. 1987; 22(2–3):273–88. https://doi.org/10.1016/0166-6851(87)90058-2 PMID: 3574347

70. Migliardo F, Tallima H, El Ridi R. Is There a Sphingomyelin-Based Hydrogen Bond Barrier at the Mammalian Host–Schistosome Parasite Interface? Cell biochemistry and biophysics. 2014; 68(2):359–67. https://doi.org/10.1007/s12013-013-9716-3 PMID: 23943053

NMR spectra of 40-49



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O
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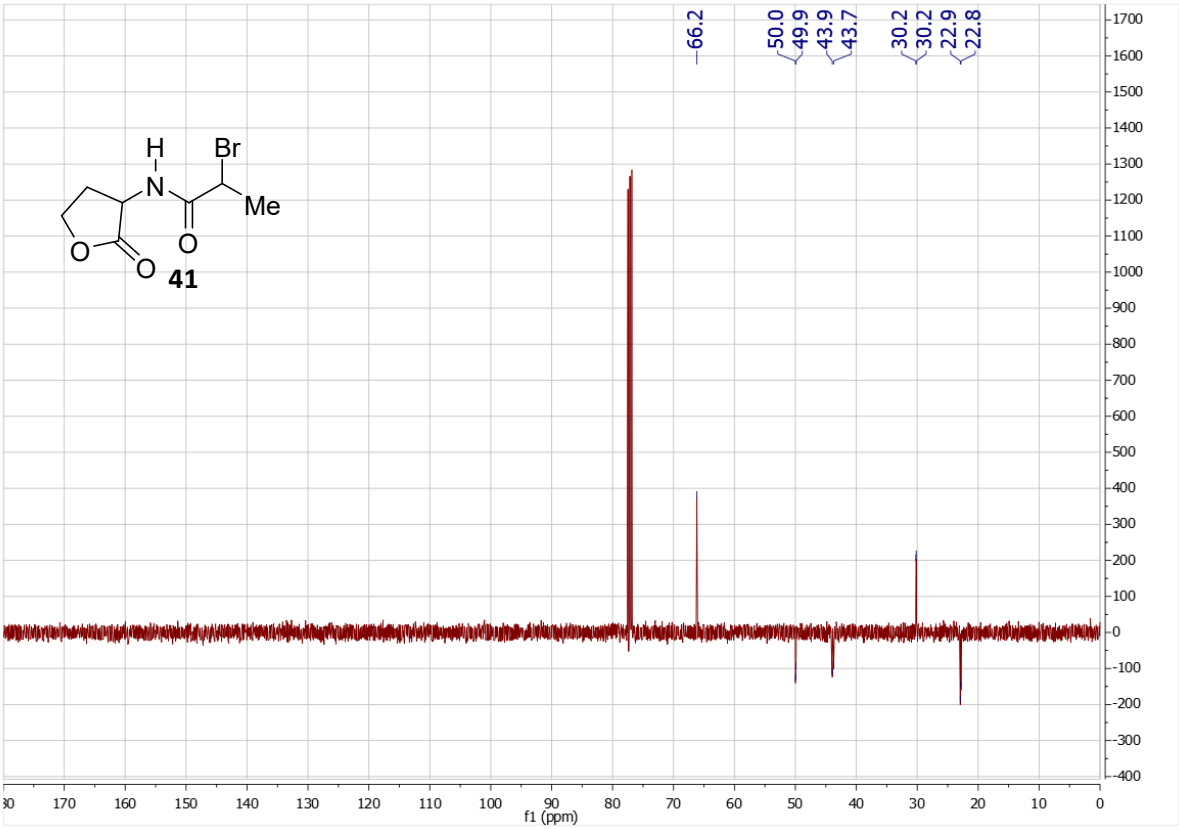
f1 (ppm)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O
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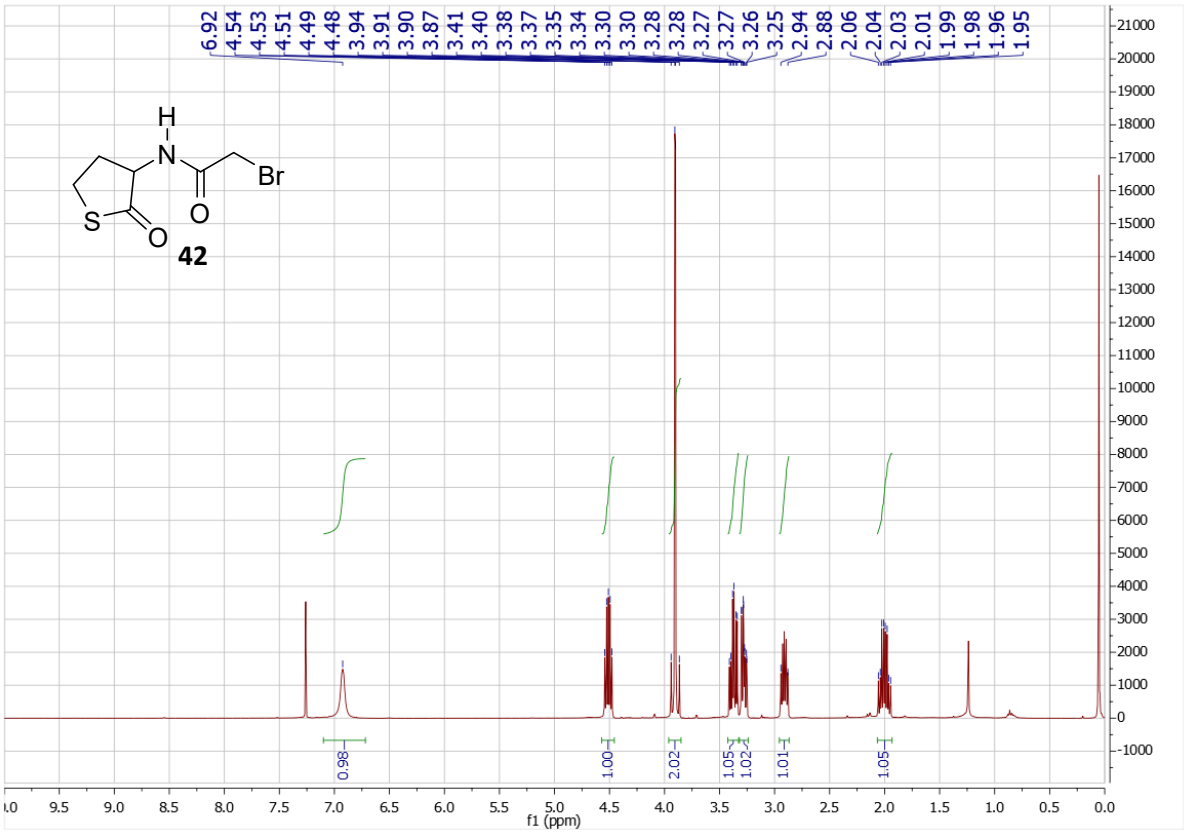
f1 (ppm)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3.37
3.35
3.34
3.30
3.30
3.28
3.28
3.27
3.27
3.26
3.25
2.94
2.88
2.06
2.04
2.03
2.01
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1.98
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1.95
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1.02
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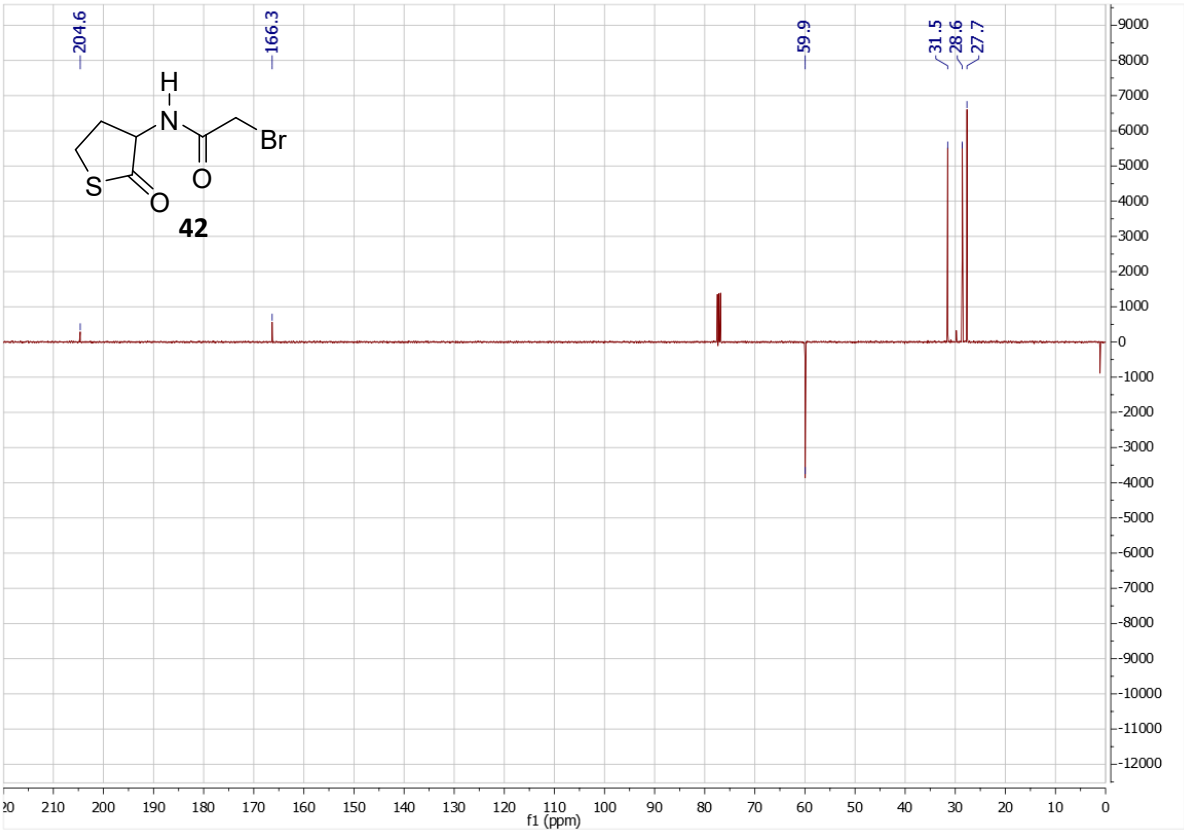
f1 (ppm)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O
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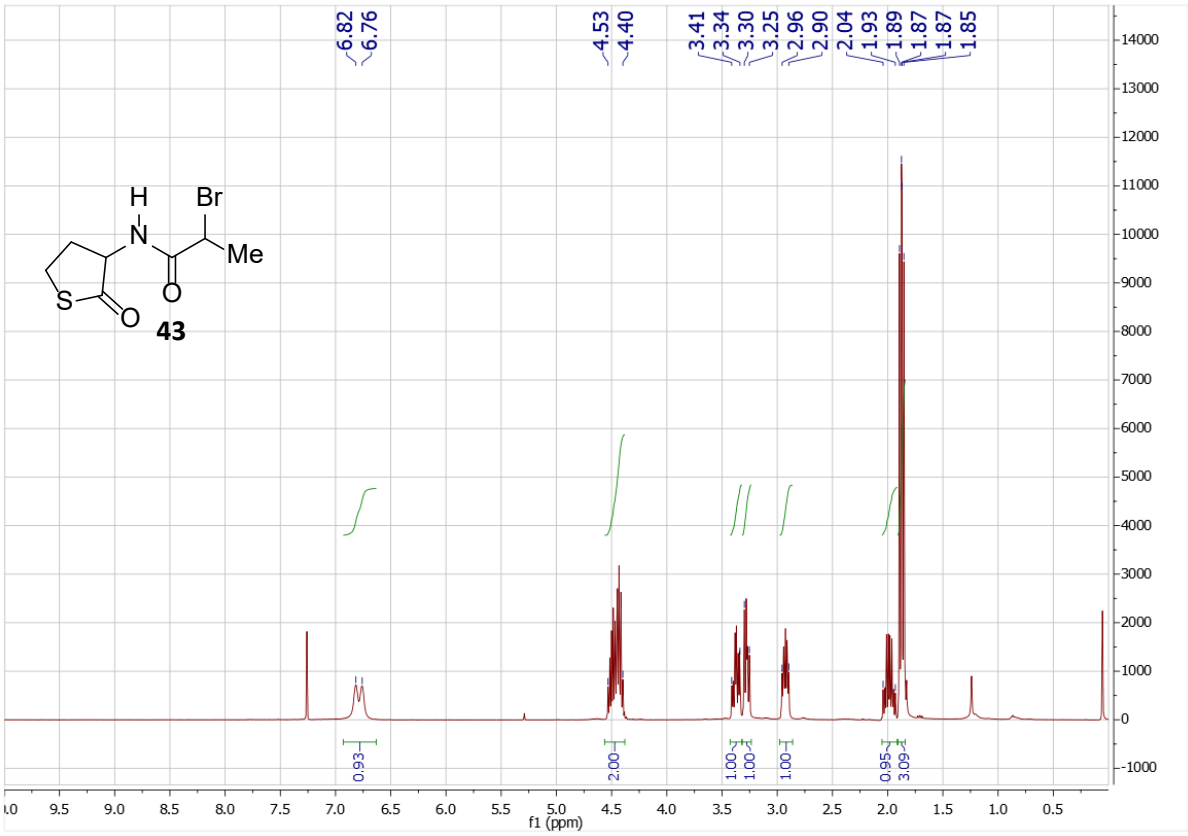
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1.87
1.87
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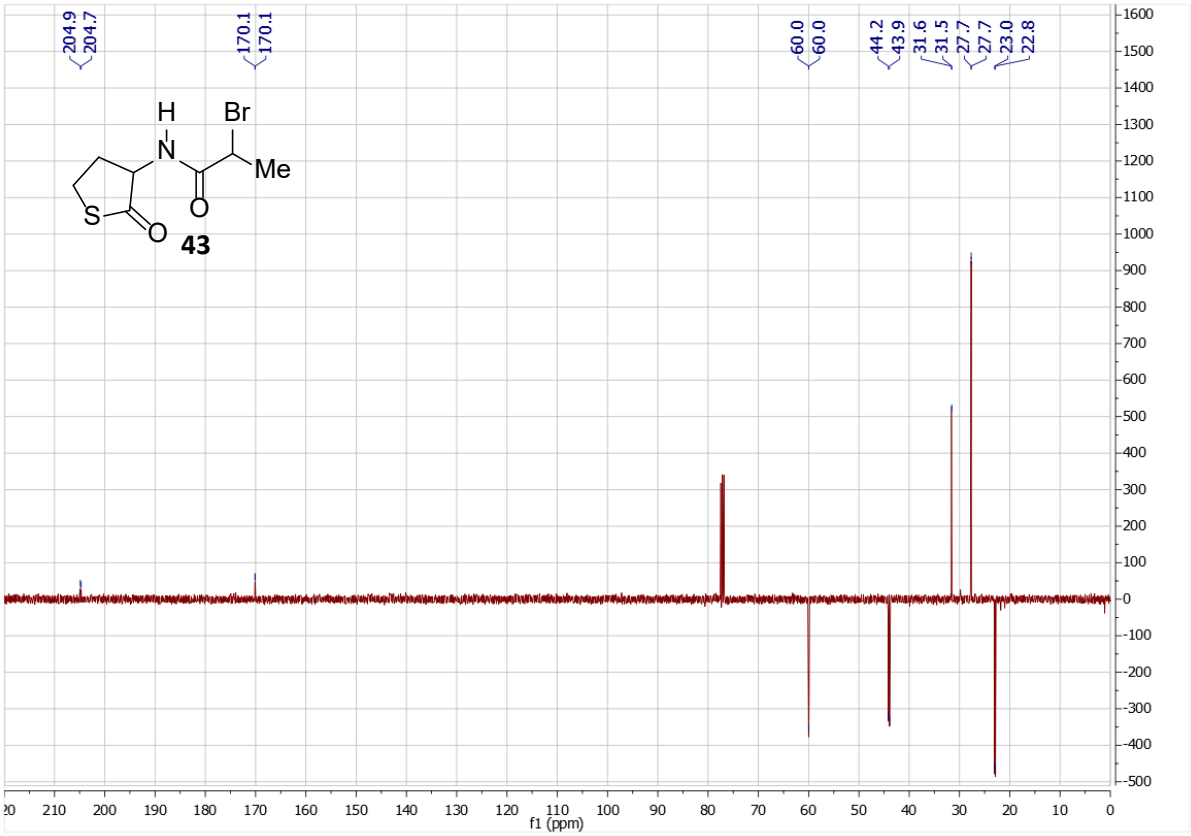
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S
O
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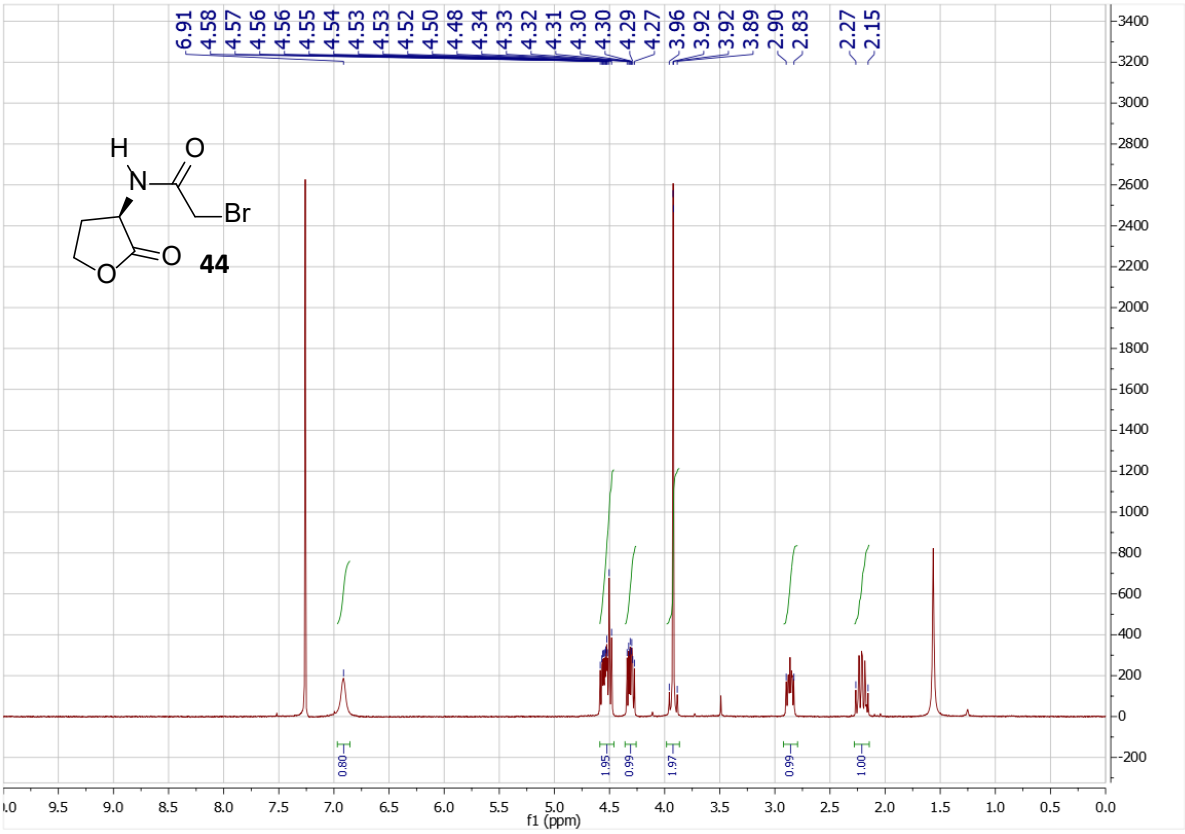
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4.30
4.29
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2.90
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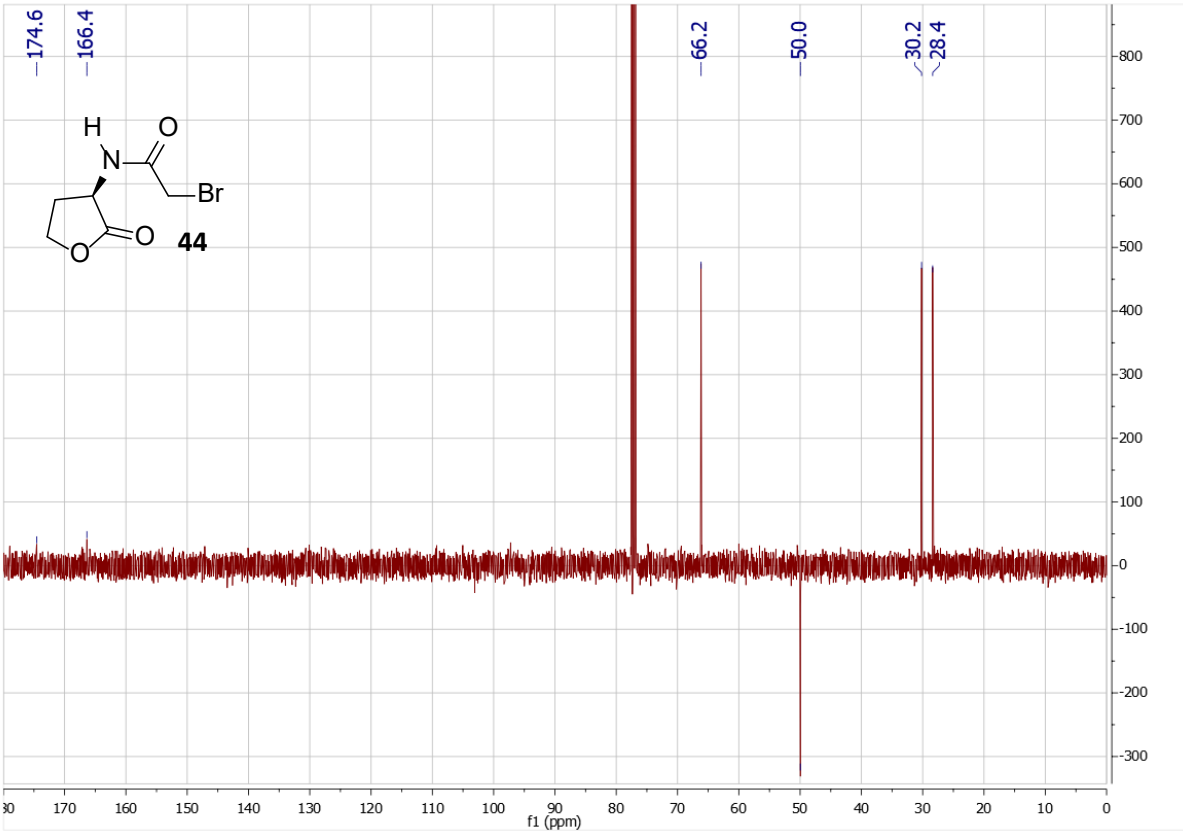
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O
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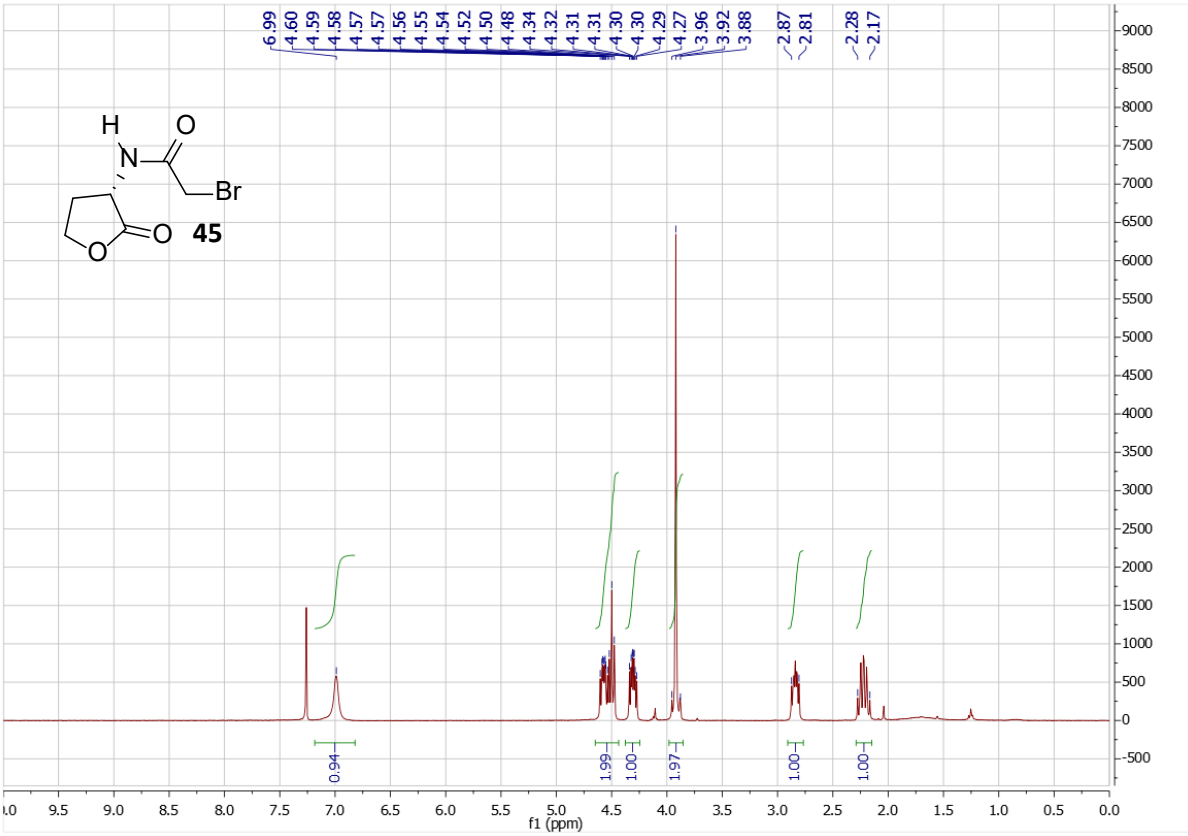
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4.30
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3.88
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2.81
2.28
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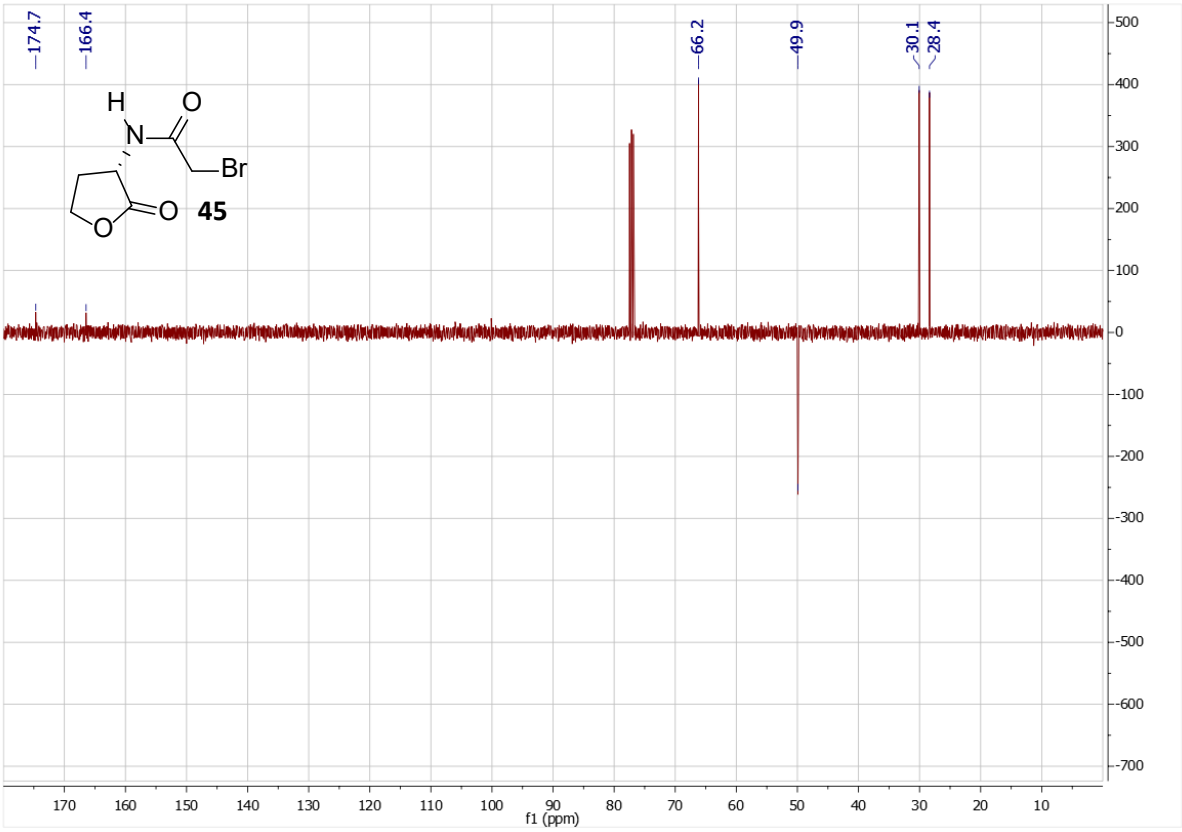
f1 (ppm)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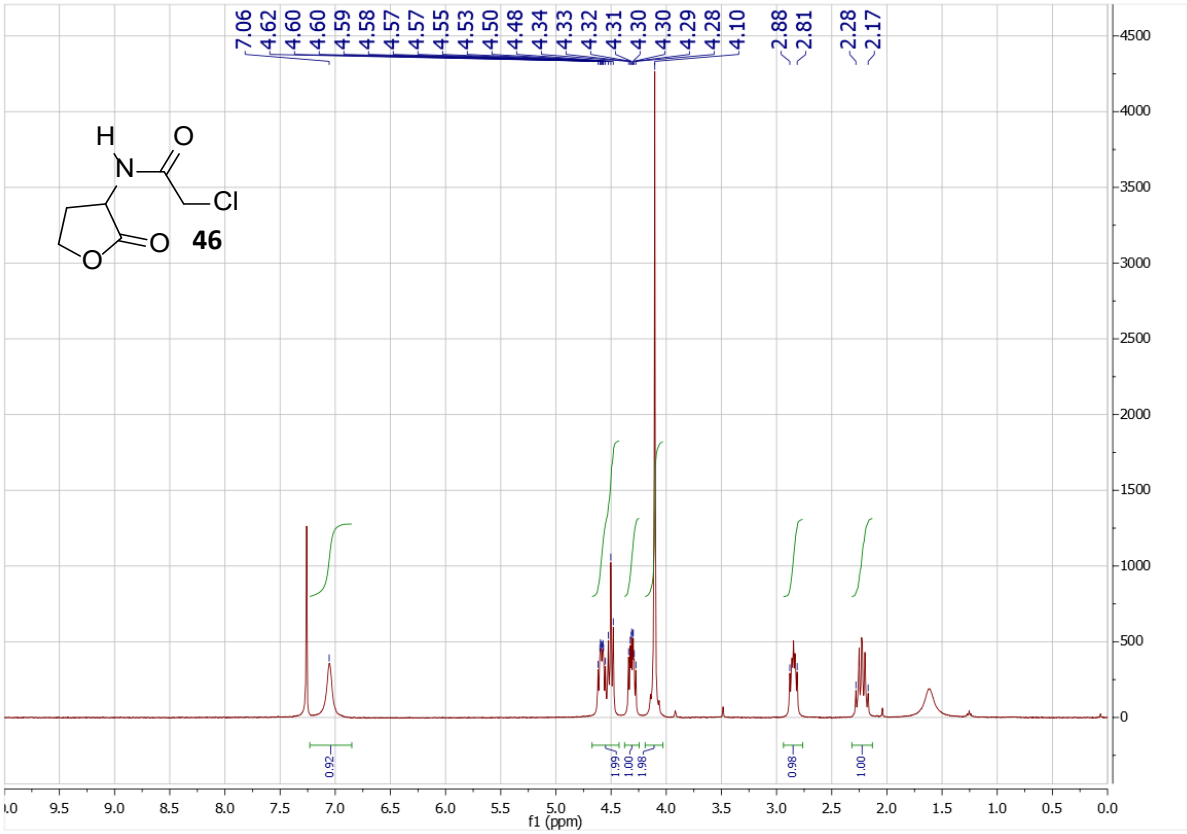
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2.28
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Cl
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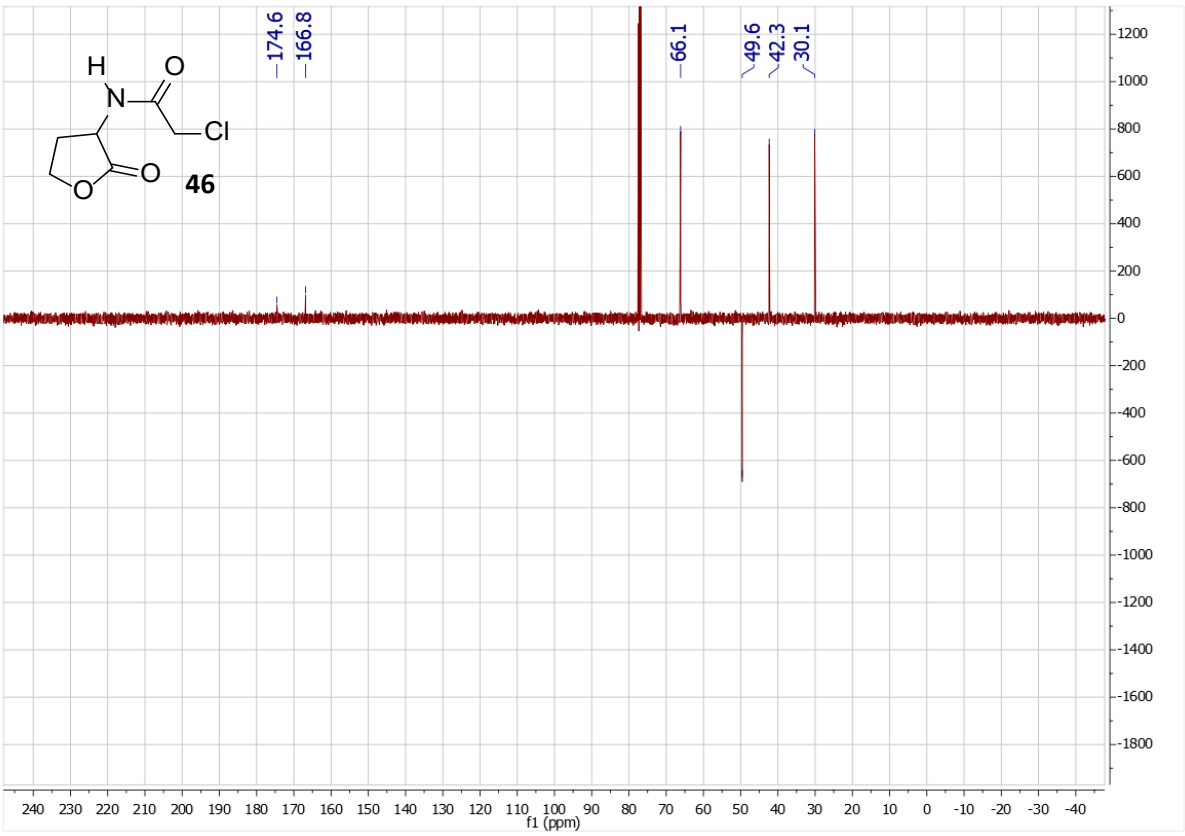
f1 (ppm)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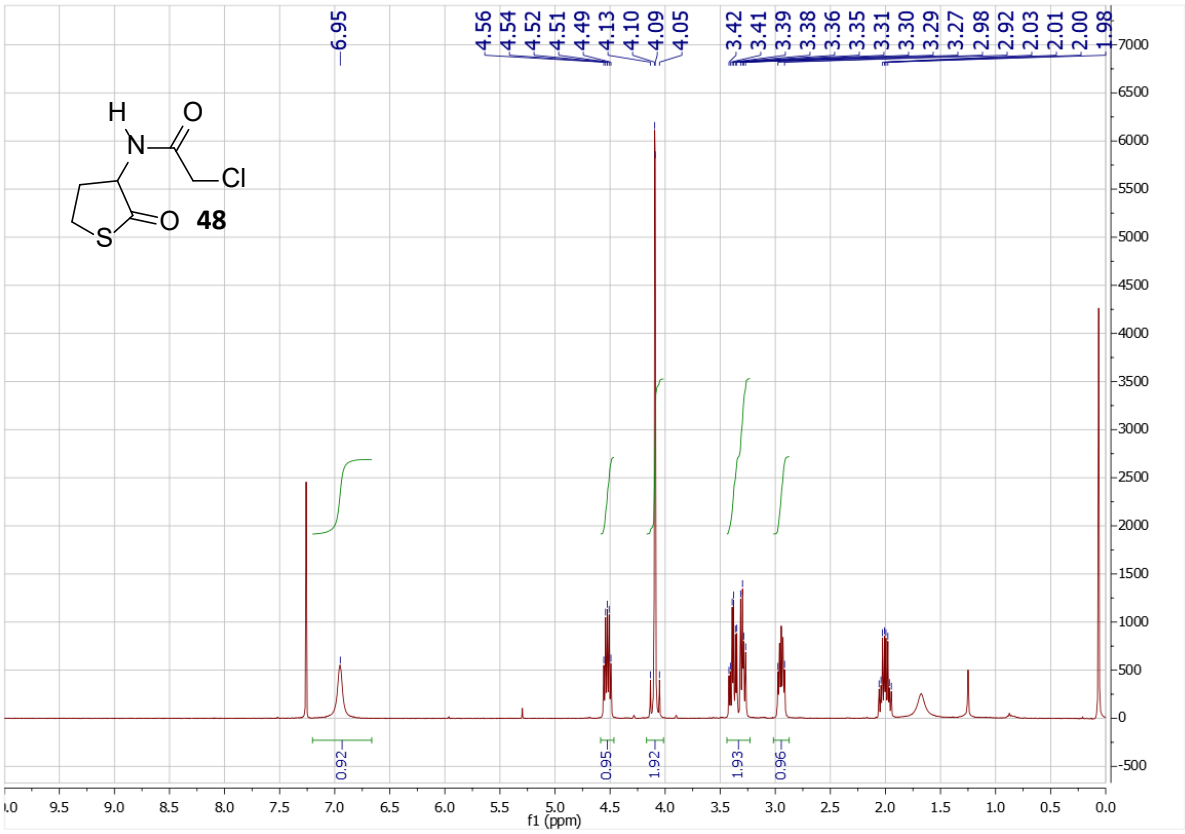
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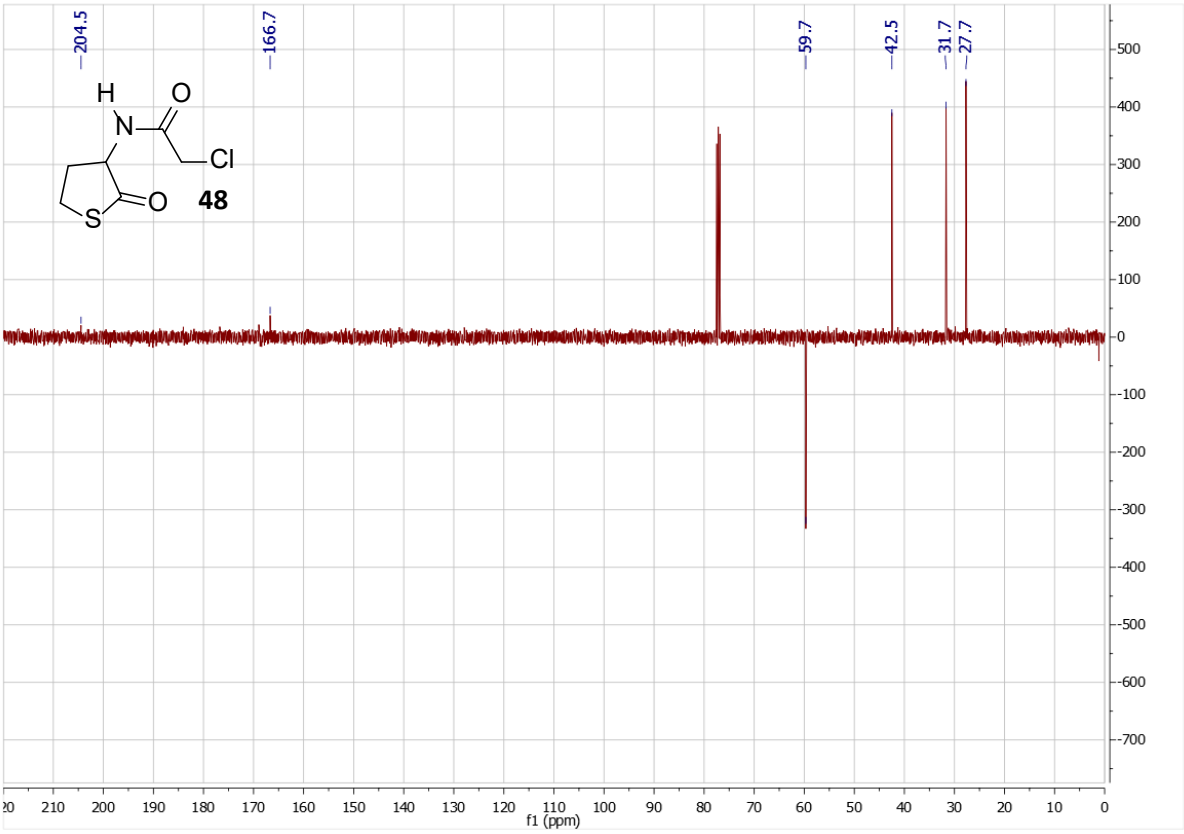
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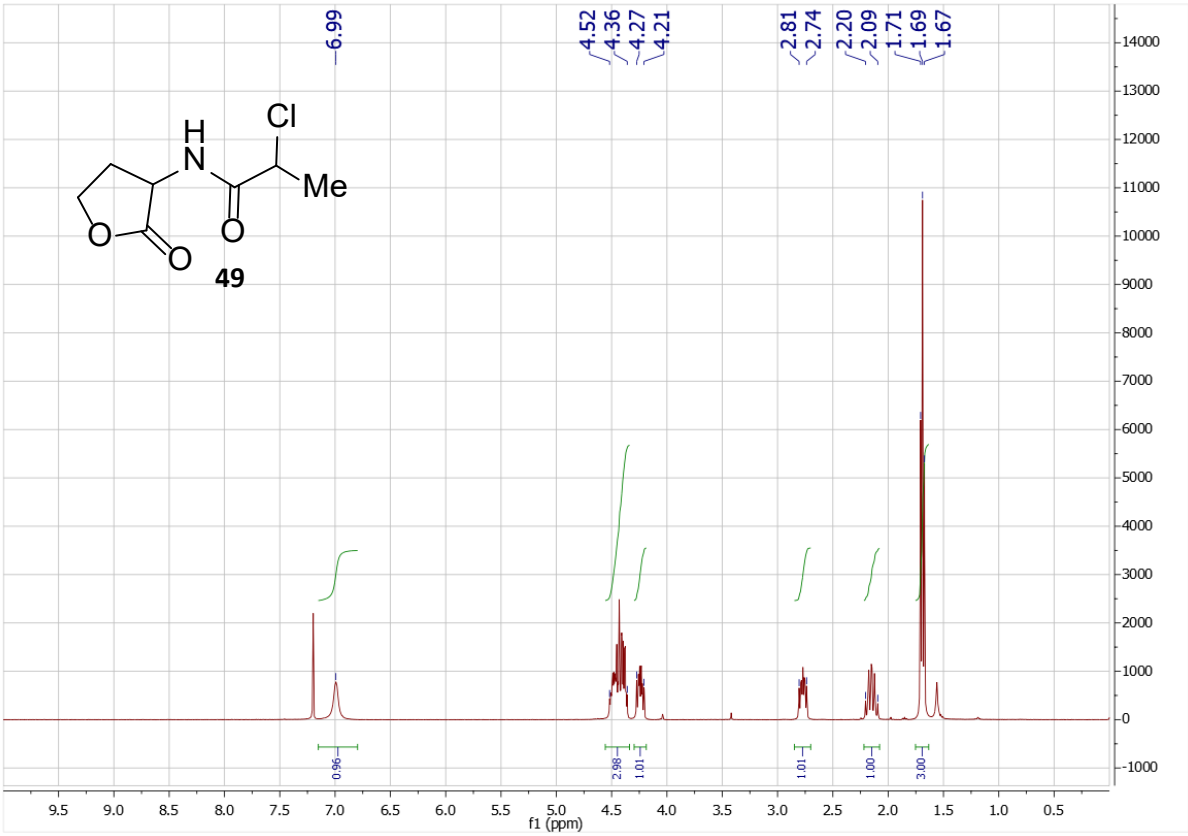
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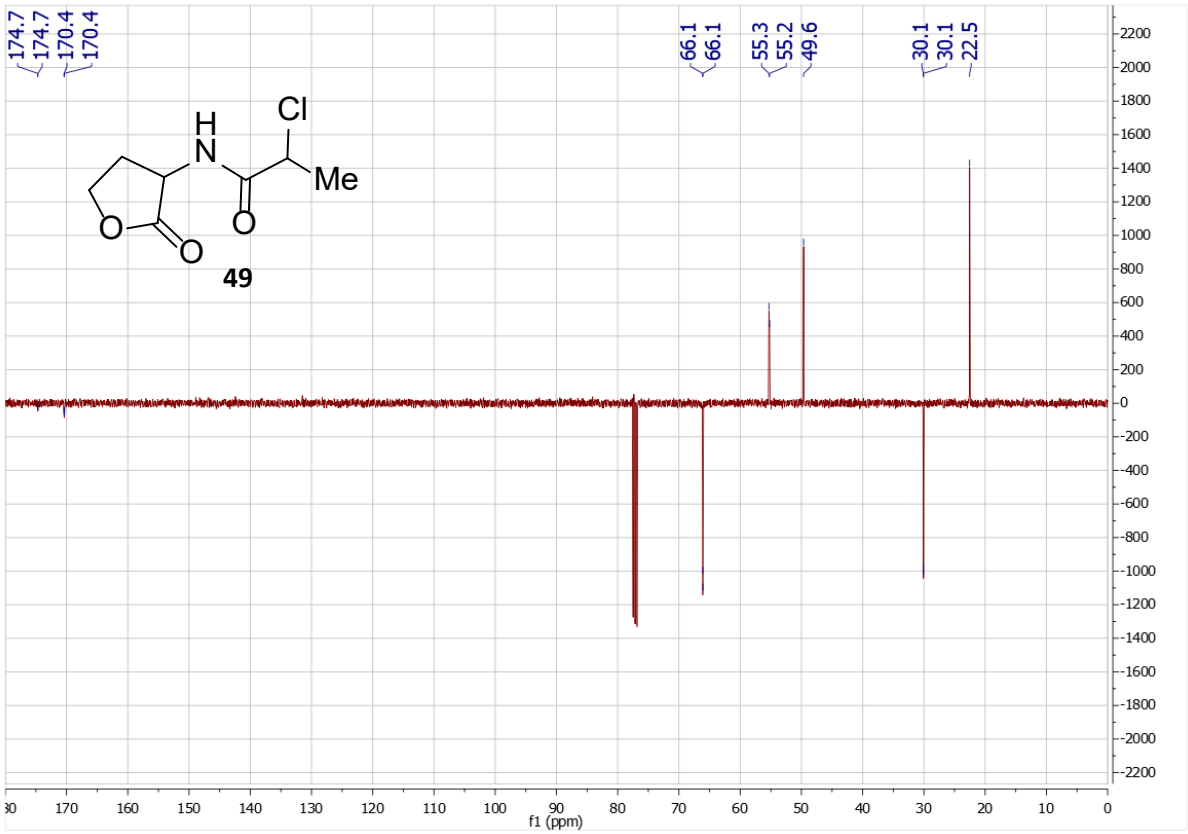
f1 (ppm)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f1 (ppm)