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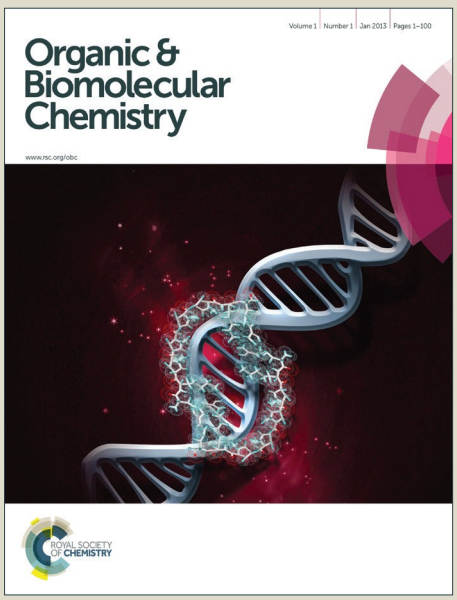

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Synthesis and Biological Evaluation of Analogs of AAL(S) for Use as Ceramide Synthase 1 Inhibitors†

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A convergent synthesis to access hydrophobic tail analogs and head group modifications of AAL(S) is described. The analogs synthesised were evaluated for their ability to inhibit ceramide synthase 1 and for their cytotoxicity in K562 cells. Our results have identified inhibitors which are non-cytotoxic yet maintain CerS1 inhibition.

Specific forms of the lipid ceramide are now well recognised as both drivers and biomarkers of insulin resistance and diabetes.[1] Ceramide synthesis is catalysed by ceramide synthases, which is a family of enzymes consisting of six isoforms (CerS1-6). Each CerS isoform catalyses the addition of a different length fatty acid group onto the amine group of sphinganine or sphingosine and this gives rise to ceramides with different biological functions.[2]

In the context of insulin resistance, it has been established that C16-ceramide (d16:1/16:0 ceramide), which is synthesised by CerS6, promotes insulin resistance in the liver,[1c,d] whilst long chain ceramides, synthesised by CerS2, are protective.[1b,d] Ceramides also promote insulin resistance in skeletal muscle,[1e] where the most abundant form of ceramide is C18-ceramide (d18:1/18:0 ceramide) synthesised by CerS1. Work in this area has been stymied due to the absence of selective and potent inhibitors of specific CerS isoforms.[3]

Interestingly, the small molecule FTY720 (**1**) has been shown to inhibit CerSs.[4] However, FTY720 (**1**) is an immunosuppressant, approved by the FDA for the treatment of multiple sclerosis.[5] It's immunosuppressive properties have been shown to arise from *in vivo* phosphorylation by sphingosine kinase 2 (SphK2) to afford the active metabolite **2**, which acts as a functional agonist at sphingosine-1-phosphate receptors (Figure 1).[6] While developing a new assay for CerS activity, we found that the chiral deoxy analog of FTY720 (**1**),

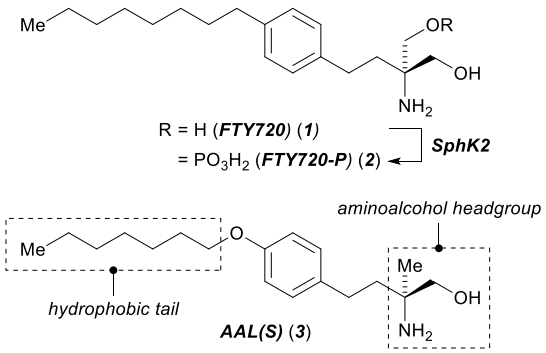

Figure 1: Structures of FTY720 and AAL(S).

AAL(S) (**3**), also has CerS inhibitory activity.[4c] In preliminary experiments we observed that AAL(S) (**3**) was reasonably potent, inhibiting the C18:0 ceramide synthase activity of CerS1-expressing HEK293 cell extracts by 50 % at a concentration of 1 μM. In contrast to FTY720 (**1**) and AAL(R),[6] AAL(S) (**3**) cannot be phosphorylated by SphK2 and as such does not have the immunosuppressive properties of FTY720 (**1**).[7,8] However, both FTY720 (**1**), in its non-phosphorylated form, and AAL(S) (**3**) are highly cytotoxic, limiting their utility as chemical probes and/or lead molecules for investigating ceramide synthase functions.[9] We therefore decided to undertake a structure-activity optimisation program with the belief that making structural modifications to AAL(S) (**3**) should allow us to engineer out the cytotoxicity, while developing a potent and selective compound that could be used to treat disease states connected to CerS1.

Previously, we found that modification to the head group and shortening of the hydrophobic tail portion of AAL(S) (**3**) can result in a marked difference in CerS activity.[4c] As such, we chose to expand on this study and synthesise analogs with modifications to the functional groups on the aminoalcohol head group and expand on our hydrophobic tail analogs with alkyl chains that are quite varied in terms of hydrophilicity and hydrophobicity.

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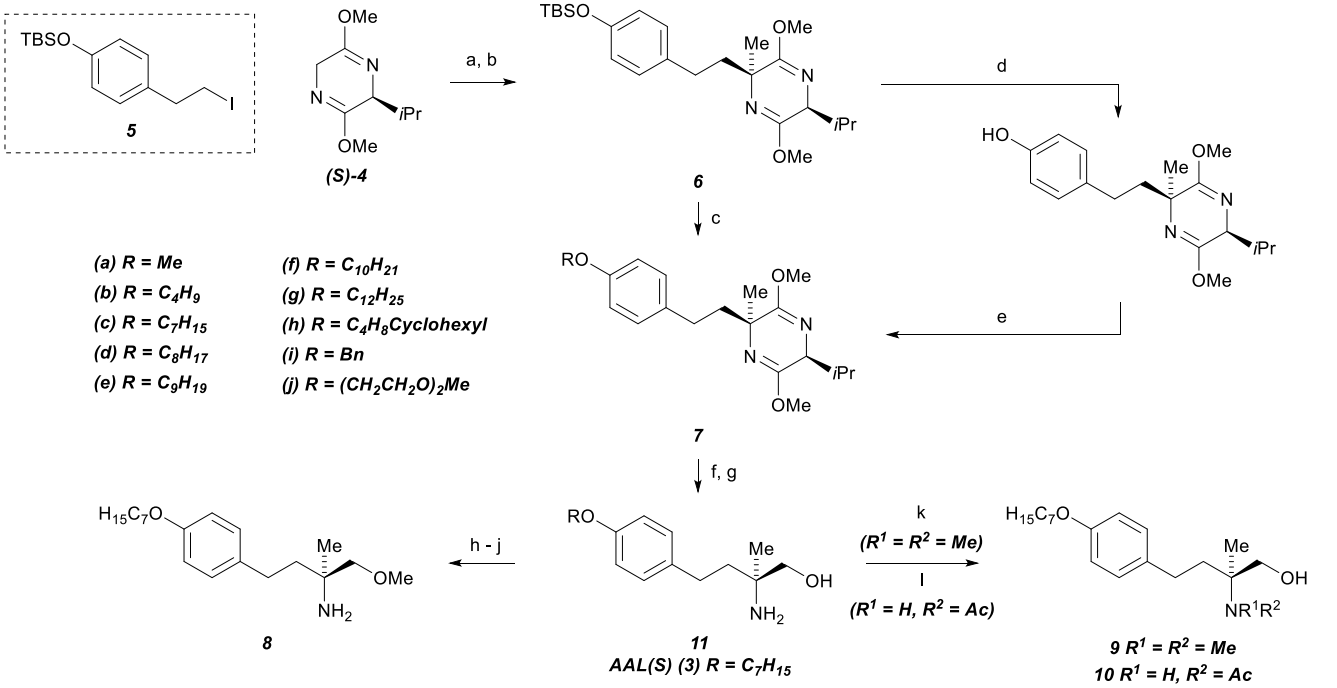

Scheme 1: *Reagents and yields* (a) *n*-BuLi, THF, -78°C, then **5**,-78°C → 0°C, 89 %; (b) *n*-BuLi, THF, -78°C, then MeI, -78°C → 0°C, 96 %; (c) CsF, RX, DMF, rt, 77 – 98 %; (d) CsF, DMF, rt, 77 %; (e) $Br(CH_2CH_2O)_2Me$, K_2CO_3, DMF, rt, 44 %; (f) TFA, H_2O, MeCN,rt, 54 – 96 %; (g) $LiAlH_4$, THF, 0°C → rt, 33 – 88 %; (h) Boc_2O, sat. aq. $NaHCO_3$, EtOAc, Δ, 83 %; (i) MeI, $(n\text{-}Bu_4N)_2SO4$, 50 % v/v 2M aq. NaOH/THF, rt; (j) 2 M aq. HCl, MeCN, Δ, 82 % (2 steps); (k) $NaCNBH_3$, $(CH_2O)_n$, AcOH, MeCN, 0°C → rt, 54 %; (l) AcCl, NEt_3, CH_2Cl_2, 0°C → rt, 35 %.

The synthesis of AAL(S) has been previously described by Hinterding and co-workers,[10] who utilised Schöllkopf's reagent to construct the aminoalcohol head group moiety in a stereoselective fashion.[11] However, in Hinterding's synthesis the heptyl tail of AAL(S) (**3**) was installed at the start. By modifying this sequence, we first developed a synthesis of AAL(S) (**3**) that would allow for convergent synthesis of a range of tail analogs. This was achieved by utilising a TBS-ether protected tail at the start so that we could employ a later stage deprotection and alkylation strategy.

Our synthesis is outlined in Scheme 1. Sequential deprotonation of (S)-Schöllkopf's reagent **(*S*)-4** with *n*-BuLi in THF at -78°C and alkylation with iodide **5** followed by alkylation with MeI constructed the quaternary stereocentre of TBS-ether **6** in 85 % yield over the two steps. Allowing the reaction mixture to slowly warm to 0°C in the -78°C cold bath was found to be critical for high diastereoselectivity. At this stage, to avoid chemoselectivity issues and protecting groups, the Schöllkopf adduct was kept intact. The TBS group was deprotected, using CsF in DMF, in the presence of 1-bromoheptane to alkylate the generated phenoxide anion.[12] This one-pot procedure generated bis-lactim ether **7** in 88 % yield. The synthesis of AAL(S) (**3**) was completed by first hydrolysis of the Schöllkopf group using TFA and H_2O in MeCN, to afford a quaternary aminoester in 87 % yield, then reduction with $LiAlH_4$ in THF in 86 % yield. This protocol yielded AAL(S) (**3**) in 56 % overall yield from iodide **5** where all spectroscopic data matched that reported in the literature.[8]

With AAL(S) (**3**) in hand, we envisaged synthesising head group analogs focussed on determining the importance of the hydrogen bond donor/acceptor capabilities of the 1-hydroxy and 2-amino functional groups. A 1-*O*-methoxy analog **8** was synthesised by first *N*-Boc protection of AAL(S) (**3**), using Boc_2O and sat. aq. $NaHCO_3$ solution in EtOAc at reflux, *O*-methylation with MeI and $n\text{-}Bu_4NSO_4$ in 50 % v/v 2M aq. NaOH/THF followed by *N*-Boc deprotection to afford 1-*O*-methyl analog **8** in 68 % overall yield for the three steps.

Two 2-amino analogs were synthesised directly from AAL(S) (**3**). The 2-*N,N*-dimethyl analog **9** could be synthesised in 54 % yield via a reductive amination protocol, using $(CH_2O)_n$ and $NaCNBH_3$ in AcOH and MeCN while the 2-*N*-acetyl analog **10** was synthesised by treating AAL(S) (**3**) with AcCl and NEt_3 in CH_2Cl_2 and in 35 % yield.

The two-step one-pot TBS-deprotection/alkylation protocol outlined for AAL(S) (**3**) allowed for the introduction of a range of tail variants by varying the alkyl halide in the reaction mixture in moderate to excellent yields (71 – 98 %) over two steps. Bis-lactim ether **7j** could not be generated via this procedure but when the two steps were carried out independently the material could be obtained in 34 % overall yield. The desired AAL(S) analogs **11** were completed by firstly hydrolysing the Schöllkopf group, in yields ranging from 54 – 96 %, and reduction of the quaternary aminoesters in 33 – 88 % yield, as described previously.

The analogs synthesised displayed variable cytotoxicity and CerS1 inhibition (Table 1).

Table 1: Comparison of the cytotoxicity of AAL(S) hydrophobic tail analogs against CerS1 activity at 10 μM.

Compound	*CerS1 Activity*[a]	*Cytotoxicity*[b]	*Selectivity*
AAL(S) (**3**)	6.0 ± 0.7	1.3 ± 0.5	0.2
8	78.0 ± 6.0	0.2 ± 0.1	0
9	25.8 ± 4.5	10.2 ± 3.6	0.4
10	83.6 ± 6.0	91.5 ± 1.1	1.1
11a	77.8 ± 5.8	89.4 ± 3.7	1.2
11b	35.1 ± 5.0	86.4 ± 5.3	2.5
11d	13.1 ± 2.8	0.6 ± 0.6	0.1
11e	10.0 ± 2.1	4.5 ± 2.5	0.5
11f	23.5 ± 5.1	21.3 ± 5.7	0.9
11g	35.0 ± 8.3	35.0 ± 4.3	1.0
11h	14.5 ± 0.8	0.9 ± 0.8	0.1
11i	24.2 ± 3.3	87.5 ± 4.9	3.6
11j	77.1 ± 5.0	91.8 ± 4.5	1.2
Control	100 ± 7.77	86.7 ± 5.2	-

[a] C18:0-NBD ceramide synthase activity of CerS1 expressing HEK293 cell extracts, in the presence of 10 μM AAL(S) analogs, 10 μM NBD-sphingosine and 50 μM C18:0-CoA. Products formed were quantified by HPLC with a fluorescent detector and the percentage of CerS inhibition normalised relative to a vehicle (water) control.[13]

[b] K562 cells were treated with 10 μM AAL(S) analogs for 24 h. Percentage viability was determined using flow cytometry on cells stained with propidium iodide. Results are mean and standard error derived from triplicate assays.

Analysis of the CerS1 data shows that none of the analogs that we synthesised were as potent as AAL(S) (**3**). In regard to the aminoalcohol head group analogs it can be observed that both the 1-*O*-methoxy analog **8** and 2-*N*-acetyl analog **10** completely lose CerS1 activity while 2-*N,N*-dimethyl analog **9** retains some activity. These results support our previous observation that compounds must retain a sphingosine-like structure (and not a ceramide-like structure as in 2-*N*-acetyl analog **10**) for competitive inhibition of the sphingosine binding pocket of the enzyme.[4c] Tail analogs with either a short methyl **11a** or hydrophilic tail **11j** were also found to be poor CerS1 inhibitors. Perhaps, not surprisingly, octyl tail analog **11d** shows comparable inhibition of CerS1 to AAL(S) (**3**), and this inhibitory activity declines as the tail becomes longer. Interestingly, the benzyl tail analog **11i** retained some inhibitory activity on CerS1.

To verify whether AAL(S) (**3**) and our synthesised analogs are cytotoxic we next tested their effect on K562 cell viability. While our results confirmed that AAL(S) (**3**) is cytotoxic they also show that head group analogs, 1-*O*-methoxy **8** and 2-*N,N*-dimethylamino **9**, which were inactive against CerS1, are highly cytotoxic. The octyl **11e** and butylcyclohexyl **11h** tail analogs are also highly cytotoxic in this assay. While the 2-*N*-acetyl analog **10**, methyl tail analog **11a** and hydrophilic tail analog **11j** displayed low cytotoxicity, their lack of activity towards CerS1 makes them insignificant. Gratifyingly however, butyl tail analog **11b** and benzyl tail analog **11i**, which displayed some CerS1 inhibition, did not reduce cell viability in comparison to the vehicle control, indicating a lack of cytotoxicity at 10 μM. The selectivity of the analogs was analysed by determining the ratio of cytotoxicity to CerS1 activity, where the higher the number the better (Table 1).

Given that the benzyl analog **11i** had the highest ratio of cytotoxicity to CerS1 activity, it was selected for further evaluation. To confirm that the compound was a selective inhibitor of CerS1, the analog was screened against the available ceramide synthases (CerS1-2, 4-6). AAL(S) was also screened as a direct comparison. As shown in Figure 2, at 10 μM AAL(S) potently inhibited CerS1, but also showed significant inhibitory activity towards CerS5 and CerS6. Benzyl analog **11i** displayed activity against CerS1, but none against any of the other CerS isoforms, indicating that CerS1 specificity is retained in this compound.

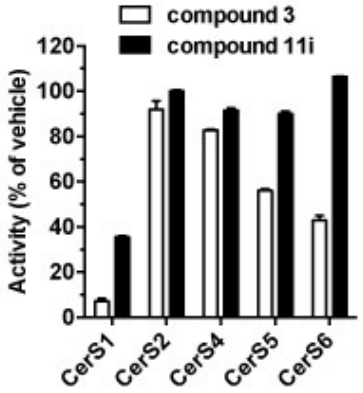

Figure 2: Selectivity of AAL(S) 3 and 11i against CerS1-2,4-6. Both compounds were tested at 10 μM on all CerS isoforms, and results are expressed as % activity relative to a vehicle (water) control. Activity was assayed using NBD-dihydrosphingosine and fatty acid-CoA as substrates, with reaction products quantified by HPLC.[13] The preferred fatty acid substrate for each CerS enzyme was used: C16:0-CoA for CerS5 and CerS6, C18:0-CoA for CerS1 and CerS4, and C24:1-CoA for CerS2.[2]

As the activity of both CerS1 and CerS4 is tested using C18 fatty acid as the substrate, this data also shows that the inhibition observed is selective for the enzyme rather than the fatty acid substrate that is used by the enzyme.

In conclusion, our results have shown that the cytotoxicity and CerS1 inhibitory of AAL(S) (**3**) can be tuned by making changes to either the aminoalcohol head group or hydrophobic tail moiety. We have found that exchanging the heptyl tail of AAL(S) (**3**) with a benzyl group allows for differentiation between the cytotoxicity and CerS1 activity. Furthermore, we have shown that the benzyl analog **11i** is selective for CerS1 over the other CerS isoforms. Thus, **11i** represents a tool molecule that could be used to study diseases connected to CerS1, such as insulin resistance.

Acknowledgements

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