Azeotropic reflux chromatography: an efficient solution to a difficult separation in the scale-up synthesis of spongistatin 1

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Azeotropic reflux chromatography (in which the eluent is continuously recycled by means of refluxing) was used to separate a mixture of spiroketal intermediates in the scale-up synthesis of spongistatin 1, leading to an improved separation and an approximately 35-fold reduction in the amount of solvent used.

Introduction

Since the seminal publication over one hundred years ago by Tswett,[1] liquid chromatography has grown in popularity and has been used in different forms to separate a great variety of compounds. Although other techniques, such as crystallisation and distillation, prove to be more efficient in many cases, they often entail some optimization and require the compounds to be either crystalline or volatile. Liquid chromatography, on the other hand, frequently provides satisfactory results with a minimum of groundwork and can, by and large, be applied to a much wider range of compounds. Indeed, in many academic research labs it has become the accepted paradigm for compound purification and separation.[2] A major disadvantage, however, is that the method requires the use of solvent, often in relatively large quantities that scale with the amount of material to be separated. Typically, the solvents used are flammable and, as such, their use is highly undesirable from a safety standpoint. In addition, there may be a significant environmental impact. Cases where the compounds to be separated elute at very similar rates generally necessitate using larger amounts of solvent. During the scale-up synthesis of spongistatin 1 (**1**), we encountered a similarly difficult separation. This led to the development of the chromatographic method that we describe herein.

Spongistatin 1 (**1**), with typical GI_{50} values of 0.025–0.035 nM when tested against the US National Cancer Institute's panel of sixty human cancer cell lines, is the most active of the spongistatin family of antitumour marine macrolides and has potential for use in anticancer chemotherapy.[3] Unfortunately, these compounds are only available in minute quantities from nature and thus total synthesis remains as the only viable source of further material. Significant interest in these molecules has led to several total syntheses being reported.[4] We recently disclosed a milligram scale total synthesis of spongistatin 1 (**1**)[5] and, confident of our synthetic strategy, we sought to repeat the synthesis on a significantly larger scale. More specifically, we aim to produce meaningful gram quantities of spongistatin 1 (**1**) for further biological and clinical evaluation.

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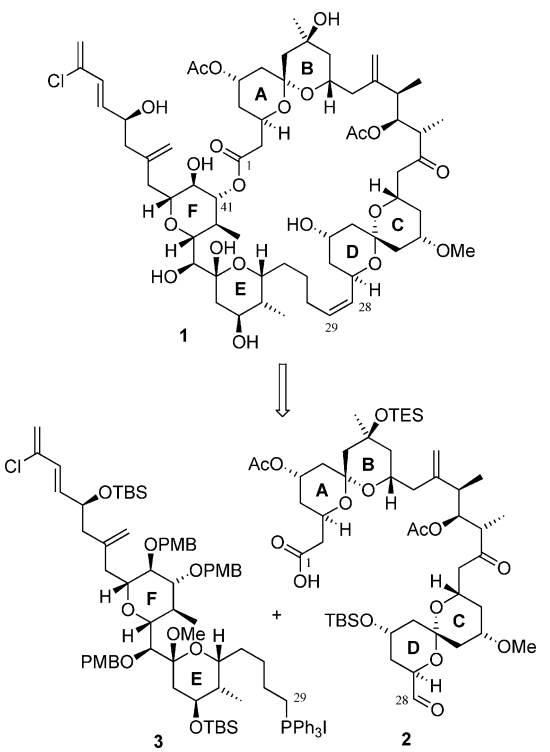
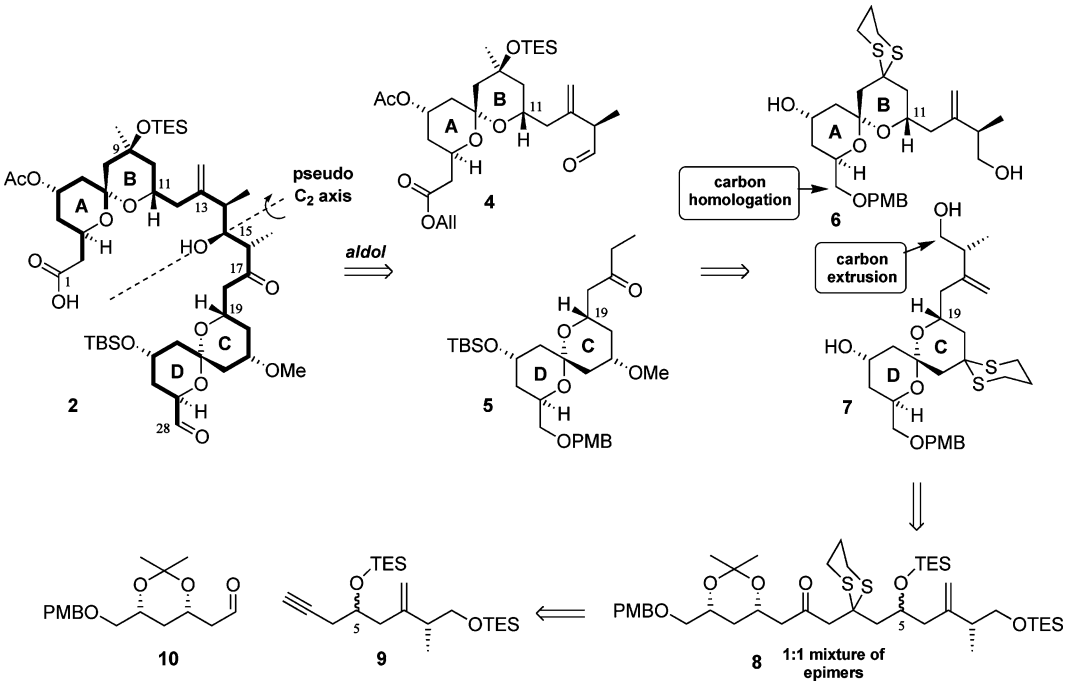
Synthetic strategy

Our retrosynthetic analysis of this molecule is based upon a late stage C28–C29 Wittig olefination and C1–C41(OH) macrolactonisation, resulting in the advanced ABCD fragment **2** and EF fragment **3**, thus allowing for a convergent synthesis (Scheme 1). Further examination of the ABCD fragment **2** reveals a high level of latent C_2-symmetry about C15 (Scheme 2). With this in mind, a synthetic route was developed in which both the C1–C15 fragment **4** and C16–C28 fragment **5** can each be differentially elaborated from the diastereomeric precursors **6** and **7** respectively. These, in turn, can both be derived from the acid catalysed deprotection–spiroketalisation of ketone **8**, which is a 1 : 1 mixture of epimers at C5 (each epimer leading to the C11 and C19 stereochemistries in **4** and **5** respectively). Thus, a single synthetic sequence can be used to supply the common intermediate. Compared with our previous

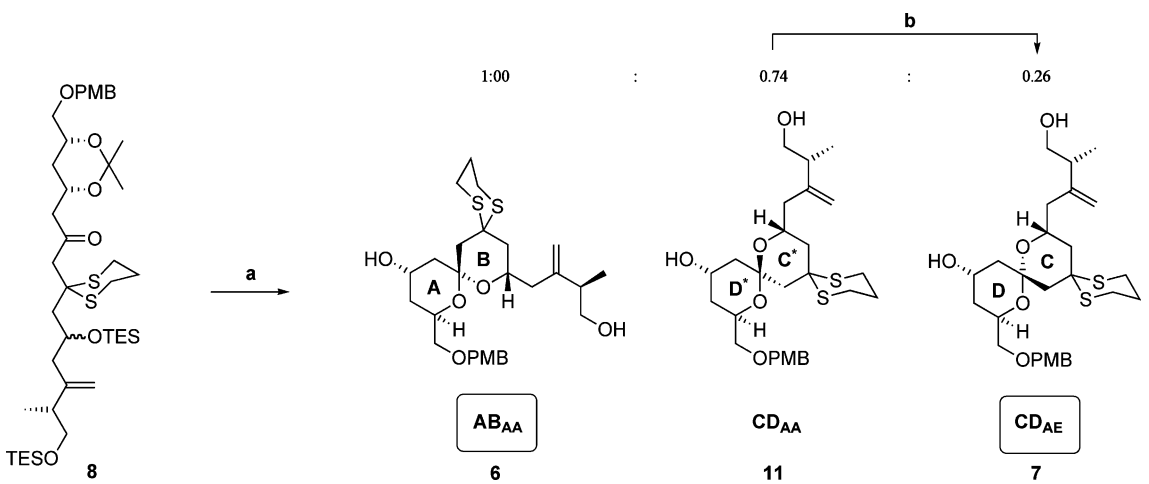


Scheme 1 Structure and retrosynthetic analysis of spongistatin 1 (**1**).



Scheme 2 Pseudo-C_2-symmetric approach to the ABCD fragment.

syntheses of the AB and CD fragments,[6] this approach is far more efficient from a scale-up point of view, dramatically decreasing the total number of steps required to construct this key unit. However, whilst this has obvious benefits, it relies on the ability to separate the very similar AB and CD spiroketal fragments prior to further elaboration. Indeed, it was recognised at an early stage that the success of the entire scale-up synthesis would hinge on this crucial separation. In the small scale synthesis it was found that treatment of ketone **8** with dilute aqueous perchloric acid in acetonitrile–dichloromethane smoothly effected cleavage of the acetonide and triethylsilyl protecting groups with concomitant cyclisation to generate three different spiroketals corresponding to the AB bis-anomeric stabilised unit **6** (AB_{AA}) and the two CD anomeric isomers, bis-anomeric **11** (CD_{AA}) and mono-anomeric **7** (CD_{AE}) in a ratio of 1.00 : 0.74 : 0.26 respectively (Scheme 3). The undesired CD_{AA} isomer **11** can easily be converted to the desired CD_{AE} spiroketal **7** under calcium perchlorate epimerisation conditions (Scheme 3). As such, **7** and **11** can be considered as a single entity in terms of separation. The key goal then is the separation of AB spiroketal **6** from the CD spiroketals **7** and **11**. During the small scale synthesis it was found that flash column chromatography could be used to efficiently separate the two CD spiroketals **7** and **11** but attempts to separate the AB spiroketal from the CD spiroketals using this technique failed. In the end, the separation was carried out using preparative HPLC (silica gel). However, the maximum loading capacity of the columns at hand (tens of milligrams) meant that multiple HPLC runs had to be performed in order to obtain sufficient quantities of separated material. This



Scheme 3 *Reagents and conditions:* (a) 10% aq. $HClO_4$, MeCN–CH_2Cl_2, r.t., 30 min, 86%; (b) 3.5% aq. $HClO_4$, 5 equiv. $Ca(ClO_4)_2{\cdot}4H_2O$, MeCN–CH_2Cl_2, r.t., 18 h, 87% after three recycles.

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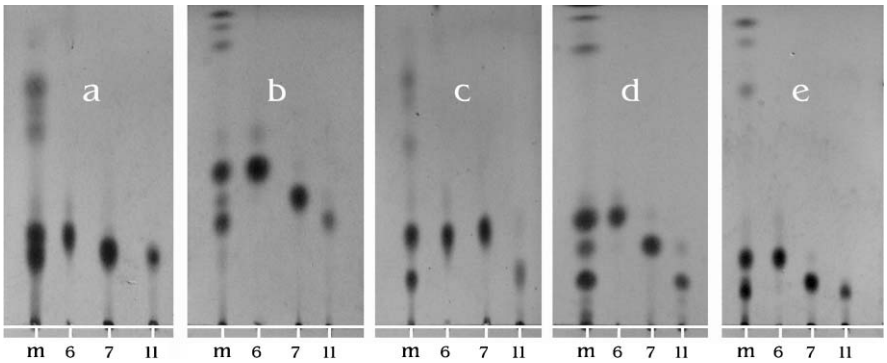

Fig. 1 TLC (SiO_2) chromatograms of the mixture (m) of **6**, **7** and **11** against reference samples. (a) Et_2O × 1 (b) 80 : 20 Et_2O–PE × 8 (c) 1 : 1 Et_2O–DCM × 1 (d) 1 : 1 Et_2O–pentane × 15 (e) DIPE × 10 (all plates stained with vanillin).

process would quite clearly be impractical for the amounts of material required for our scale-up synthesis (*ca.* 40 g each of AB_{AA} (**6**) and CD_{AE} (**7**)). Rather than spend time investigating the use of expensive, high-capacity HPLC columns, attention turned to the reinvestigation of traditional silica-gel column chromatography.

In order to determine a suitable solvent for flash chromatography, TLC studies were carried out on a variety of binary and ternary solvent systems. Unfortunately, no combination led to the resolution of the three spiroketals into individual spots on the chromatogram. An example chromatogram (neat diethyl ether) is shown in Fig. 1a. It can be seen that the spots streak and tend to merge into each other. Multiple-elution TLC (whereby the processes of elution and drying are repeated several times prior to development) was then attempted in an effort to afford resolution. Of the solvent mixtures initially assayed, those based on diethyl ether–petroleum ether were found to provide the best resolution. Shown in Fig. 1b is a chromatogram of the crude spiroketal mixture run 8 times in 80 : 20 diethyl ether–petroleum ether. Three separate spots, corresponding to each spiroketal, can easily be seen. Solvents based on the diethyl ether–DCM system led to a greater separation of **7** and **11** (even in single elution) but no separation between **6** and **7**. Pleasingly, when the diethyl ether–petroleum ether solvent system was used in preparative TLC, three resolved bands were obtained (using UV light to visualise) from which analytically pure (^{1}H NMR) samples could be extracted.

Column chromatography

Having established a potential solvent system, small scale column chromatography was attempted. After some experimentation, the use of a solvent gradient was found to give optimal results. For example, the crude product from reaction of 40 mg of the ketone **8** was loaded onto 7 g of silica gel (packed using neat petroleum ether) with 1 mL 1 : 1 DCM–petroleum ether and was eluted with 500 mL 1 : 1 diethyl ether–petroleum ether collecting 10 mL fractions. The column was then eluted with a solvent gradient from 1 : 1 diethyl ether–petroleum ether to 70 : 30 diethyl ether–petroleum ether over 800 mL. At this point all of the AB_{AA} (**6**) and CD_{AE} (**7**) had eluted. The remainder of the CD_{AA} (**11**) was then eluted with 300 mL diethyl ether. The fractions were combined into four samples: 10 mg clean **6**, 3 mg 2 : 1 **6** : **7**, 2 mg clean **7**, 8 mg clean **11** corresponding to an 89% yield for the reaction and 83% separation of **6**. As these small scale studies afforded reasonable levels of separation, a larger scale separation was attempted. The crude product from reaction of 24 g of ketone **8** was loaded onto 2 kg of silica gel (packed in petroleum ether) with 30 mL 1 : 1 : 1 DCM–diethyl ether–petroleum ether. The column was then eluted with 50 L 1 : 1 diethyl ether–petroleum ether, collecting fractions of 500 mL. A solvent gradient was then started from 1 : 1 diethyl ether–petroleum ether to diethyl ether over 150 L, after which time all the AB_{AA} (**6**) had eluted. The solvent was then drained and the column flushed with acetone until all the CD_{AA} (**11**) had eluted (11 L). After combination of fractions three samples were obtained: 6.19 g clean **6**, 2.68 g 1 : 1 : 1 **6** : **7** : **11**, 5.35 g 1 : 8 **7** : **11**. This corresponds to a 92% yield and an 87% separation of the AB_{AA} spiroketal **6**. Although this level of separation was acceptable, two major issues were encountered. Firstly, the amount of solvent used for the column was well over 200 L, which is unacceptable in terms of cost, safety and environmental concerns. Secondly, the time spent running the column (*i.e.* refilling the solvent reservoir, changing fraction vessels and evaporating solvent) was extremely long (over 40 h). As significantly greater amounts of material would need to be purified, an alternative, more efficient method was sought for the separation of these spiroketals.

We envisaged that a system in which the solvent is continually recycled by means of reflux, thereby combining the properties of the chromatography column with those of the Soxhlet extractor, might lead to a dramatic reduction in solvent volume and operating effort. Whilst this concept had been demonstrated on a limited number of other examples,[7] it had not been used to separate stereoisomers. Additionally, we were concerned about the stability of our compounds to prolonged heating after elution and therefore wished to investigate the use of relatively low-boiling solvents, including low-boiling azeotropic mixtures (which, to our knowledge, have not been employed previously). To test the applicability of the general concept on a small scale, we fashioned a prototype apparatus using equipment to hand (Fig. 2a), which operates as follows: the solvent in the collecting round bottomed flask is heated to reflux with stirring and the vapour travels up the glass tubing at the side of the column, the condensate collects on the top of the column through which it travels on its way back to the round bottomed flask. Of course, the difference in elution times of the compounds to be separated has to be greater than the length of time collecting each fraction to avoid remixing in the collecting vessel. As the solvent cycle involves reflux, this would severely limit

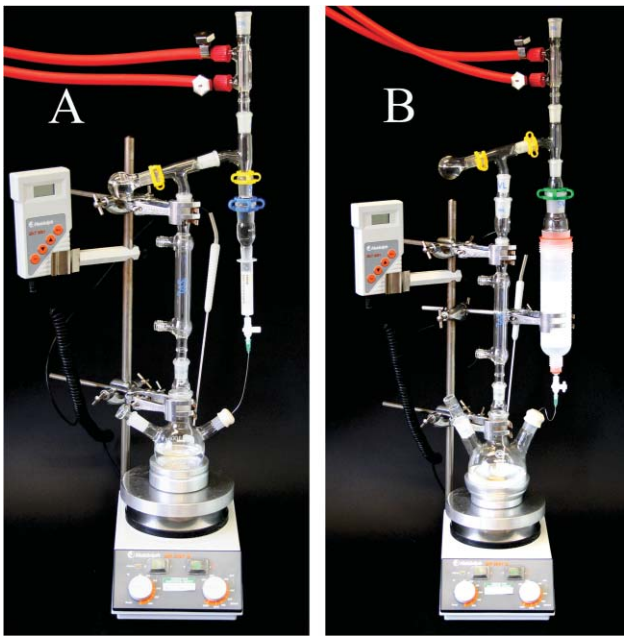

Fig. 2 Prototype reflux chromatography apparatuses.

the solvent system used due to the phenomenon of azeotropic distillation. In order to keep the eluting solvent isochratic we could either employ a single solvent or use a low-boiling azeotrope with the required properties. Both options were investigated. To simplify matters, we abandoned the use of petroleum ether as this is an ill-defined mixture of hydrocarbons whose composition may vary from batch to batch.

Diethyl ether was initially retained as the polar component. Hexane, which is a constituent of petroleum ether and has comparable chromatographic qualities, could not be used as the non-polar partner as it fails to form an azeotrope with diethyl ether. Instead, we considered pentane, which is inexpensive and forms an azeotrope with diethyl ether of almost 1 : 1 composition (b.p. 34 °C).[8] TLC studies supported this choice. Shown in Fig. 1d is a multiple-elution chromatogram of the mixture of spiroketals eluted 15 times in 1 : 1 diethyl ether–pentane before development. As an alternative to an azeotrope we also considered the use of a less polar dialkyl ether. In terms of boiling point and cost/availability, two obvious options were methyl *tert*-butyl ether (MTBE, b.p. 56 °C) and diisopropyl ether (DIPE, b.p. 69 °C). Whilst MTBE afforded insufficient separation in TLC studies, DIPE seemed to be the solvent of choice. As shown in Fig. 1e, when eluted 10 times in DIPE, the CD_{AE} (**7**) spot runs much closer to that of the CD_{AA} (**11**) spiroketal and the separation between the former and the topmost AB_{AA} (**6**) spot is higher. Using the diethyl ether–pentane azeotrope, we conducted a small scale trial of the process using the apparatus shown in Fig. 2a. Crude spiroketal mixture resulting from reaction of 50 mg of ketone **8** in 2 mL 1 : 1 : 1 diethyl ether–DCM–pentane was loaded onto 2 g silica gel (loading: 25 mg g^{-1}), which was packed with 1 : 1 diethyl ether–pentane. The 100 mL round bottomed flask was charged with 80 ml 1 : 1 diethyl ether–pentane and heated to reflux and collection began. The contents of the collecting flask were monitored by TLC analysis. After 3 h only spots higher than the AB_{AA} (**6**) (mainly due to triethylsilyl byproducts) were present; the solvent was changed and collection continued. The AB_{AA} (**6**) material then began to elute. At 6 h, slight traces of CD_{AE} (**7**) were observed by TLC and the solvent was changed once more. At 7 h, no AB_{AA} (**6**) was observable, only CD_{AE} (**7**) and CD_{AA} (**11**). The column was flushed with acetone (20 mL) and these

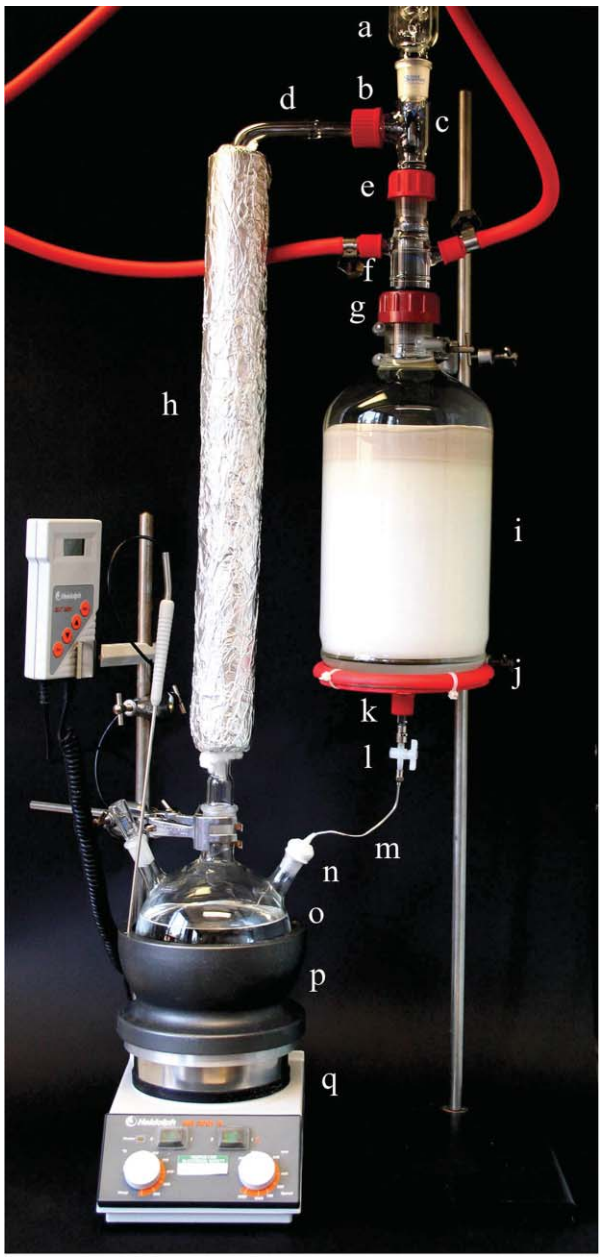

Fig. 3 (a) 20 cm jacketed coil condenser; (b) screw-thread connector (GL 25); (c) side-arm adapter (23 mm o.d.); (d) L-shape glass tubing (15 mm o.d.); (e) Rodaviss™ B24 ground glass joint; (f) 80 mm jacket condenser (B34 male cone incorporated into jacket); (g) Rodaviss™ B34 ground glass joint; (h) side-arm and insulation (5 mm cotton wool within 10 mm polystyrene insulating foam covered with aluminium foil); (i) column, 15 cm o.d., height 19 cm (excluding taper), porosity 3 sinter; (j) retaining ring; (k) screw-thread connector (GL 14, lined with PTFE tape) to cut-down luer-lock 2 mL glass syringe; (l) polythene luer-luer tap; (m) 150 mm 14 G stainless steel syringe; (n) B14 latex suba-seal (syringe and seal wrapped with PTFE tape and Parafilm®); (o) 1 L 3-neck round bottomed flask; (p) aluminium heating block; (q) stirrer-hotplate.

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flushings were combined with the 6 h–7 h fraction. After removal of solvent, two fractions were obtained: 16 mg 10 : 1 **6** : **7** (3 h–6 h), 14 mg 1 : 3 **7** : **11** (6 h–7 h plus the acetone flush material) corresponding to a 92% yield. Although the separation was not complete, we felt this could be improved by collecting fractions at shorter intervals. The same experiment was also carried out using DIPE as solvent. A similar level of separation was observed but, in this case, the material obtained seemed to be impure by NMR analysis. This degradation may have been due to the higher boiling point of DIPE (69 °C). This, added to the fact that DIPE has a noted tendency to form explosive peroxides,[9] led us to abandon its use and we settled on the diethyl ether–pentane mixture for all subsequent work. Using the slightly larger apparatus shown in Fig. 2b, the separation was carried out with crude spiroketal mixture from reaction of 1.5 g of ketone **8**. In this case, 46 g of silica gel (loading: 33 mg g^{-1}) was used and three fractions were obtained: 425 mg **6**, 55 mg 5 : 3 **6** : **7**, 412 mg 1 : 3 **7** : **11**. This corresponds to a 92% reaction yield and 93% separation of the AB_{AA} spiroketal (**6**). Having established that the concept was applicable, we designed and built[10] a larger apparatus that would allow for the separation of significant quantities of the spiroketal materials (Fig. 3). In previously reported systems, where very few fractions were collected, the column is placed vertically above the solvent reservoir/collector, connected to it by a ground glass joint.[7] We felt that this would make the process of changing solvent fractions difficult. As we intended to collect several fractions, we kept the configuration of our prototype system in which the column is held separately, horizontally removed from the solvent reservoir/collector but attached to it by connections that are easily removed during solvent change. A second condenser (Fig. 3f) was included, between the column and side arm adapter (Fig. 3c), to provide cooling to the solvent condensate. The column was designed to hold at least 1 kg of silica gel and was relatively wide to provide sufficient solvent flow through the porosity 3 sinter as well as to keep the total height of the apparatus within reasonable working limits. The volume between the sinter and the luer outlet was the minimum that could be achieved within the practical limits of construction. This diminished any post-column remixing of eluates. The side arm was lagged with a layer of cotton wool inside insulating foam covered with aluminium foil. With this in place, a sufficient rate of reflux of the diethyl ether–pentane azeotrope could be maintained with the heating block at around 100 °C. The flow rate from the column packed with 1 kg of silica and solvent at the level of the side arm adapter was an acceptable 80 mL min^{-1}.

On this larger scale, a slightly higher loading (42 mg g^{-1}) of material was used. The crude product from reaction of 42 g of ketone **8** in 45 mL 1 : 1 : 1 DCM–diethyl ether–pentane was loaded onto 1 kg of silica gel packed with 1 : 1 diethyl ether–pentane. After the solvent had been adsorbed, the silica gel was covered with a 2 inch layer of sand (low in iron) and 1 : 1 diethyl ether–pentane was added up to the level of the side-arm adapter. The 1 L round bottomed flask was charged with 800 mL 1 : 1 diethyl ether–pentane and heated to reflux. After solvent had started to condense above the column, the tap at the bottom was opened and elution began. The contents of the collecting flask were monitored periodically by TLC analysis. Fractions were collected on average every 7 h. Fraction collection involved turning off the heating, allowing the heating block to cool until reflux had stopped (about 5 minutes), closing the luer-luer tap, disconnecting the screw thread connector (Fig 3b) and hypodermic needle (Fig 3m), removing the side arm (Fig 3d) and changing the solvent in the round bottomed flask before reversing the process. In total, the time needed to collect each fraction was around 10 minutes. The AB_{AA} (**6**) began to elute at 21 h (fraction 4, Fig. 4). Prior to this, the triethylsilyl byproducts had completely eluted. The CD_{AE} (**7**) started to co-elute with the AB_{AA} at 58 h (fraction 11, Fig. 4). After 93 h (fraction 16, Fig. 4), the last traces of AB_{AA} (**6**) had eluted and the CD_{AA}

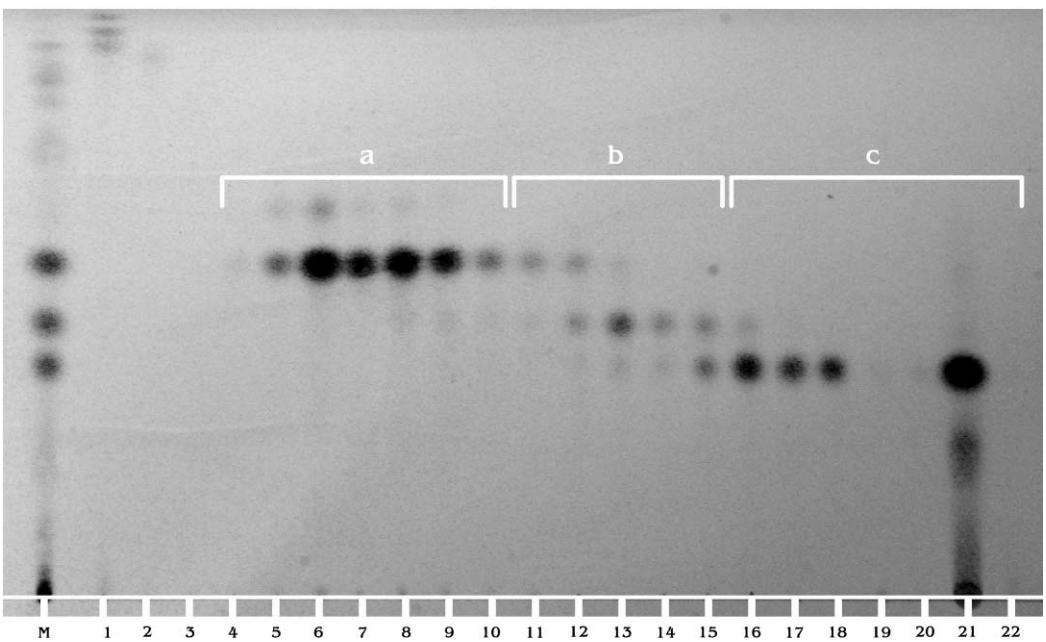

Fig. 4 TLC chromatogram of fractions from reflux chromatography against reference a mixture (M) of **6**, **7** and **11**. Eluted with 10 × 80 : 20 Et_2O–petroleum ether (stained with vanillin solution).

(**11**) was the major component of the eluate. After 124 h (fraction 19, Fig. 4), the CD_{AA} (**11**) appeared to have completely eluted by TLC analysis. At this point the reflux was stopped and the column was allowed to drain under gravity and then flushed with 3 L of acetone. Interestingly, the acetone washes (fractions 19–22, Fig. 4) contained significant quantities of the CD_{AA} spiroketal (**11**). After combination of fractions and evaporation of solvent, three samples were obtained: 11.40 g **6**, 3.74 g 26 : 58 : 16 **6** : **7** : **11**, 10.12 g **11** (a, b and c in Fig. 4). This corresponds to a 93% reaction yield and a 92% separation of AB_{AA} (**6**). The total amount of solvent used was 17 L. Using the measured flow rate of 80 mL min^{-1}, the amount of solvent required to run the same column in the traditional manner is estimated to be in excess of 590 L. Thus, the requisite volume of solvent has been significantly reduced, by a factor of around 35. Not only does this signify a considerable reduction in cost, but it also represents a substantial lessening of the environmental impact of the process. Furthermore, as our solvent system is an azeotropic mixture, it has the same composition after distillation and can be collected from the rotary evaporator after product isolation to be reused in subsequent separations. This idea is currently being employed as we continue our scale-up synthesis of spongistatin 1 (**1**).

Conclusion

Our route to spongistatin 1 (**1**) exploits the high level of C_2 pseudosymmetry present in the ABCD fragment **2**, and relies on the ability to separate the AB_{AA} fragment **6** from the CD fragments **7** and **11**. We have developed a novel system which is cost-effective and uses a very simple apparatus to continuously recycle the azeotropic solvent mixture. The method allows for a dramatic reduction in the amount of solvent required, uses no expensive equipment and is easy to construct and operate.

We suggest that this little-used technique could have a much broader application in organic synthesis programmes in the future.

Acknowledgements

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