Dynamic combinatorial chemistry with hydrazones: cholate-based building blocks and libraries†

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We describe an efficient and general strategy for the synthesis of dimethyl acetal functionalised steroidal hydrazides based on the cholic acid skeleton with the aim of using these compounds as building blocks for dynamic combinatorial chemistry. Deprotection of the acetal protected building blocks with TFA leads to formation of libraries containing macrocyclic *N*-acyl hydrazone oligomers. The isolation of several of these, and their characterisation using NMR is described. The effects on the equilibrium library distribution by varying the substituents at C-7 and C-12, extending the side-chain with glycine, and inverting the configuration at C-3 are discussed. Finally, we report the exchange properties of these macrocycles and demonstrate new examples of proof-reading and self-sorting in dynamic combinatorial libraries.

Introduction

The ability of a template molecule to direct the assembly of, or to select, its 'ideal host' by means of non-covalent interactions is fundamental to the success of catalytic antibodies,[1] ribozymes[2] and their DNA equivalents[3] as well as certain imprinted polymers.[4] In terms of producing catalysts and molecular hosts capable of recognition all have their attendant advantages and disadvantages.[5]

In the past decade, dynamic combinatorial chemistry has emerged as a powerful new templating approach for the discovery of unexpected supramolecular systems.[6] In a dynamic combinatorial library (DCL), a series of receptors is allowed to interconvert in solution through reversible bond-making and bond-breaking. The reversibility gives the system the possibility to respond to the addition of a template in such a way as to reveal stabilising non-covalent intermolecular interactions. An equilibrium distribution of receptors should be perturbed by the addition of a template that binds selectively to, stabilises and hence favours the formation of one particular receptor. The DCL can be screened *in situ*, presenting a very attractive way of avoiding the laborious and difficult task of designing molecular hosts or guests. Elegant examples of template-directed assembly under thermodynamic control have been reported, leading to the discovery of new receptors and ligands utilising a variety of different reversible chemistries.[7–9]

The results reported herein build on our early work using transesterification in DCLs, where we reported a series of macrocyclic receptors based on cholate building blocks, whose equilibria can be perturbed by the addition of alkali metal ions.[10]

In our search for a mild alternative to transesterifcation, to permit the use of organic templates and foster weak non-covalent intermolecular interactions, we have been actively investigating the trifluoroacetic acid catalysed exchange behaviour of macrocyclic *N*-acyl hydrazones;[11] the acid catalysed exchange of macrocyclic imines was reported by Wild around the same time as this work began.[12] Historically, *N*-acyl hydrazones were for many years used for isolating and characterizing liquid aldehydes and ketones. For other applications, they have been largely neglected in the chemical literature. This is surprising, given their appealing characteristics: they are stable, easily accessible from carbonyl compounds and hydrazides, and they possess potential pseudo-peptidic recognition features (Fig. 1).[13]

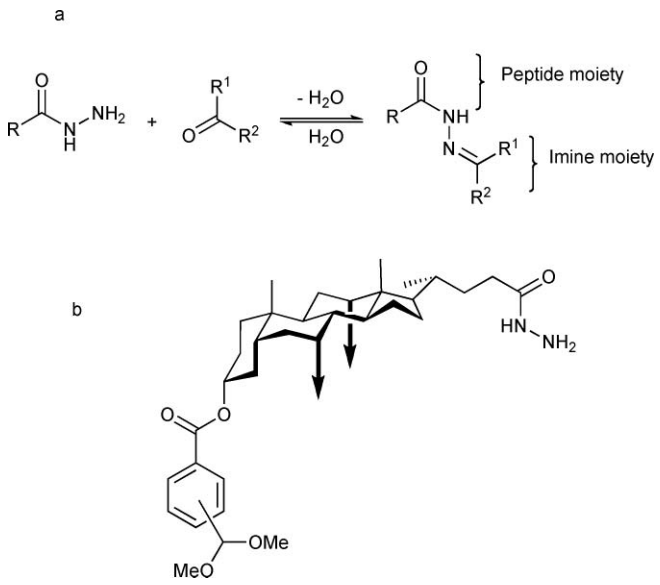

Fig. 1 a) The reversible hydrazone reaction. b) General design of cholate-derived building blocks.

The pedigree of bile acids as supramolecular building blocks for recognition has been well established. Davis[14] and

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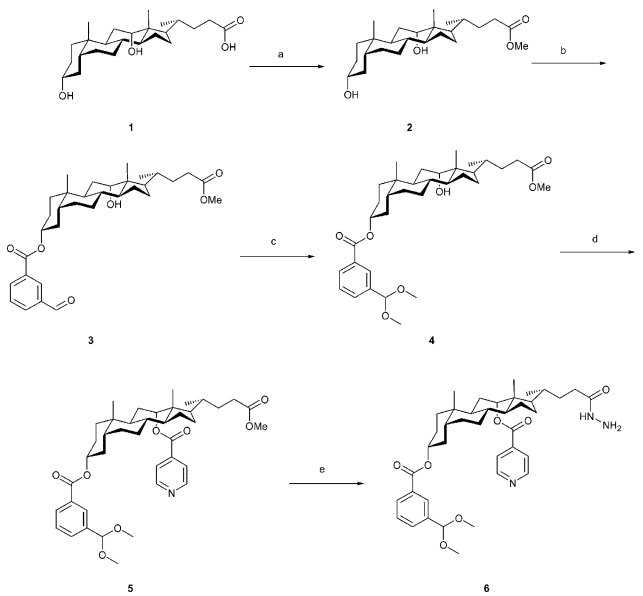
Bonar-Law and Sanders[15,16] have shown that these molecules can be functionalised and cyclised to form rigid cavities, capable of recognising carbohydrates, alkaloids and metal ions, either alone, or as part of other supramolecular architectures. The purpose of the work described here was to equip a range of functionalised bile acids with a dimethyl acetal and a hydrazide and study both the range and nature of the macrocyclic *N*-acyl hydrazones formed upon deprotection with a view to thermodynamic templating. This work builds on our earlier studies of steroid[16] and amino acid-based[17] macrocyclic *N*-acyl hydrazones.

Results and discussion

Synthesis

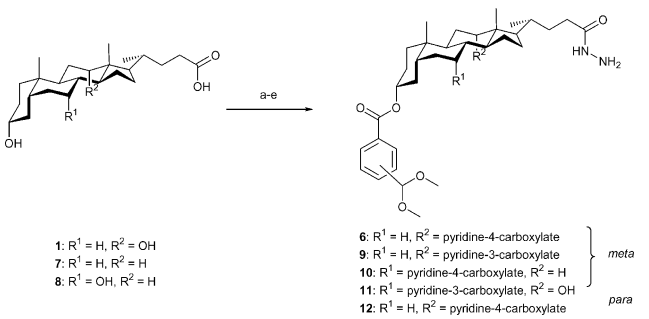
Lithocholate, deoxycholate, chenodeoxycholate and cholate-based dimethyl acetal functionalised hydrazides were obtained by applying, with modifications where appropriate, the synthetic methodology shown in Scheme 1. Methyl deoxycholate (**2**) was synthesised, using the method of Fieser, in 85% yield from deoxycholic acid (**1**).[18]



Scheme 1 Synthesis of building blocks exemplified by the deoxy series. Reagents and conditions: a) MeOH, AcCl, room temperature. b) 3-Carboxybenzaldehyde, EDC, DMAP, CH_2Cl_2. c) *p*TsOH, MeOH. d) Pyridine-4-carboxylic acid, EDC, DMAP, CH_2Cl_2. e) NH_2NH_2, MeOH, room temperature.

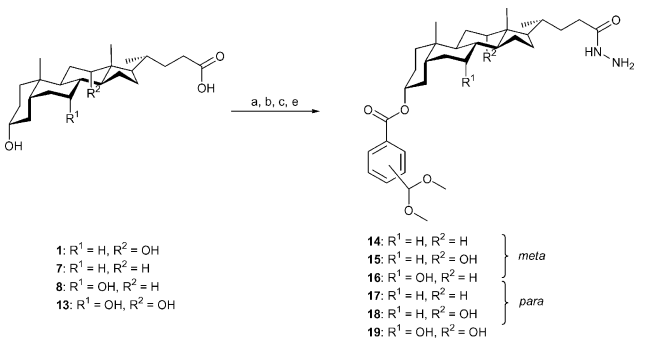
Selective esterification of the diol (**2**) at the 3-position was achieved by means of EDC mediated coupling of 3-carboxybenzaldehyde in CH_2Cl_2, with a catalytic amount of DMAP, to form the ester (**3**) in 85% yield.[19] The aldehyde (**3**) was protected as its dimethyl acetal (**4**) by means of a catalytic amount of *p*TsOH in MeOH. Coupling of pyridine-4-carboxylic acid at the 12-position of **4** to form the ester (**5**), in 90% yield, was also achieved by means of EDC in CH_2Cl_2. This required a stoichiometric amount of DMAP, however, compared to the catalytic amount sufficient for coupling at the 3-position. Efficient and selective hydrazinolysis of the methyl ester (**5**) to form the hydrazide (**6**) in 90% yield required 30 equivalents of hydrazine monohydrate, and a concentrated solution of **5** in MeOH or EtOH at room temperature.[20] The reaction typically required 48 h to reach completion. Heating was avoided, due to the presence of the aromatic esters at the 3- and 12-positions of the steroid, which react under forcing conditions.

The data in Scheme 2 summarises the pyridine-substituted steroidal hydrazides that have been prepared by the general reaction sequence depicted in Scheme 1, substituting reagents where appropriate. Thus, the synthesis of **9** required the use of pyridine-3-carboxylic acid, while **10** and **11** were synthesised from chenodeoxocholic acid (**8**). In order to investigate the effect of changing the geometry of a dimethyl acetal functionalised hydrazide on the range of macrocyclic *N*-acyl hydrazones formed at equilibrium 3-carboxybenzaldehyde was substituted for 4-carboxybenzaldehyde in the synthesis of **12**.



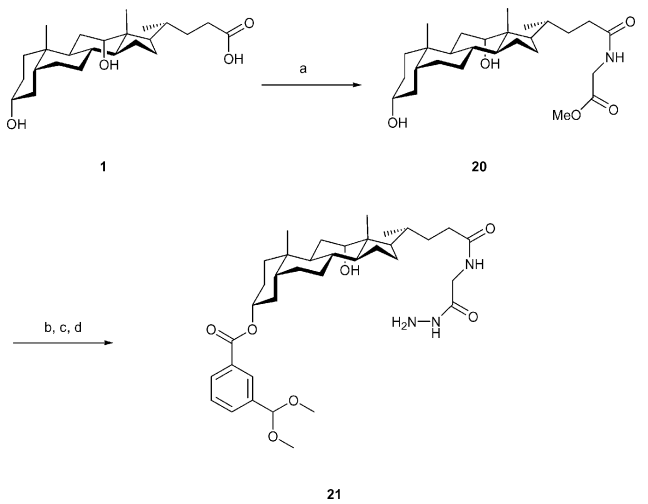
Scheme 2 Summary of pyridine-appended building blocks. Reagents and conditions as in Scheme 1.

A second series of steroidal hydrazides, depicted in Scheme 3, does not possess pyridine-3 or 4-carboxylate substituents at the 7- or 12-positions of the steroid. The aim with this sequence of molecules was to leave free hydroxyl groups, to potentially enable recognition and binding of polyhydroxylated guests, such as carbohydrates.[21] Again 4-carboxybenzaldehyde was substituted for 3-carboxybenzaldehyde in the synthesis of **17**, **18** and **19**. As well as being derived from deoxycholic (**15** and **18**), and chenodeoxycholic acid (**16**), one monomer (**19**) was synthesised from cholic acid. The lithocholate derivatives (**14** and **17**) were to be used as control molecules.



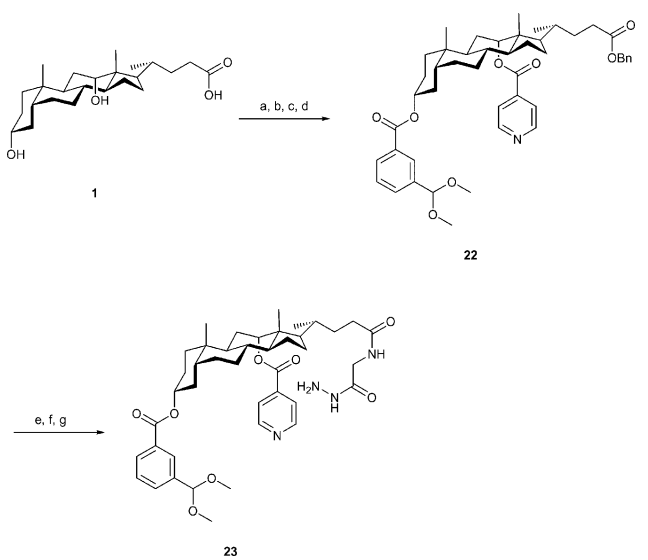
Scheme 3 Second series of building blocks. Reagents and conditions: See Scheme 1.

To further investigate the effect of changing the geometry and flexibility of a dimethyl acetal functionalised steroidal hydrazide on the range of macrocyclic *N*-acyl hydrazones formed at equilibrium the glycine extended steroidal hydrazide (**21**) shown in



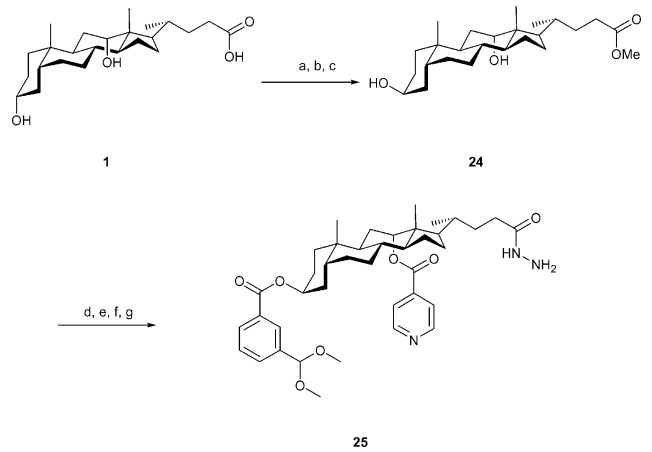
Scheme 4 Synthesis of extended steroid building blocks. Reagents and conditions: a) Glycine methyl ester, EDC, DMAP, Et_3N, room temperature. b) 3-Carboxybenzaldehyde, EDC, DMAP, CH_2Cl_2. c) *p*TsOH, MeOH. d) NH_2NH_2, MeOH, room temperature.

Scheme 4 was synthesised. The methyl ester (**20**) was obtained in 95% yield following EDC mediated coupling of glycine methyl ester hydrochloride with **1** in CH_2Cl_2, using a catalytic amount of DMAP and Et_3N. The alcohol (**20**) was selectively esterified at the 3-position with 3-carboxybenzaldehyde by means of EDC and a catalytic amount of DMAP in CH_2Cl_2. The aldehyde was protected as the dimethyl acetal by treatment with a catalytic amount of *p*TsOH in MeOH. Finally hydrazinolysis of the methyl ester with 20 equivalents of hydrazine monohydrate in MeOH, afforded the hydrazide (**21**) in 56% overall yield. The pyridine-appended extended building block (**23**), was prepared in 43% overall yield from the carboxylic acid (**1**) by the closely-related route summarized in Scheme 5.



Scheme 5 Synthesis of extended steroid building blocks. Reagents and conditions: a) Benzyl alcohol, EDC, DMAP. b) 3-Carboxybenzaldehyde, EDC, DMAP, CH_2Cl_2. c) *p*TSOH, MeOH. d) Pyridine-4-carboxylic acid, EDC, DMAP, CH_2Cl_2. e) Pd/C 10%, H_2. f) Glycine methyl ester, EDC, DMAP, Et_3N. g) NH_2NH_2, MeOH, room temperature.

The final variation in structure was to invert the configuration at the 3-position of deoxycholic acid (Scheme 6). This was achieved by the previously reported method by Fiegel *et al.*[22] Mitsunobu coupling of formic acid with the alcohol (**2**) using DIAD and PPh_3 in THF led to the 3β formate ester in 85% yield.[23]



Scheme 6 Synthesis of steroid building block with inverted stereochemistry. Reagents and conditions: a) MeOH, AcCl, room temperature. b) Formic acid, DIAD, PPh_3, THF. c) Na, MeOH, room temperature. d) 3-Carboxybenzaldehyde, EDC, DMAP, CH_2Cl_2. e) *p*TsOH, MeOH. f) Pyridine-carboxylic acid, EDC, DMAP, CH_2Cl_2. g) NH_2NH_2, MeOH, room temperature.

Removal of the formate group was achieved using a freshly prepared solution of sodium methoxide to afford the alcohol with the stereocentre reversed (**24**) in 75% yield from the carboxylic acid (**1**). The subsequent steps were identical to those employed in the synthesis of the hydrazide (**6**) leading to the hydrazide (**25**) in 25% overall yield from the ester (**2**).

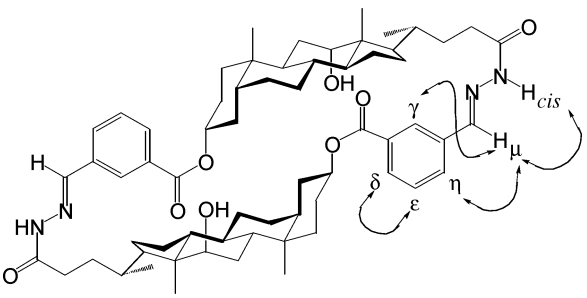
Cyclisation studies

In order to favour the formation of macrocyclic *N*-acyl hydrazones over linear polymers, deprotection of the dimethyl acetal functionalised hydrazides was conducted under high dilution conditions.[24] Thus, a 5 mM solution of the monomer in CH_2Cl_2, $CHCl_3$ or DMSO was treated with 5% TFA v/v at room temperature. The acid serves to remove the dimethyl acetal protecting group and also to catalyse formation of the macrocyclic *N*-acyl hydrazones. In CH_2Cl_2 and $CHCl_3$, deprotection of the dimethyl acetal and cyclisation to form the *N*-acyl hydrazones were complete in a matter of minutes, while the reaction in DMSO had to be stirred at room temperature for 24 h before all of the starting material had been consumed. For the purposes of recognition and isolation of macrocycles we were more interested in, and so focused upon, deprotection in the chlorinated solvents.

Analysis of the reaction mixtures, directly by reverse phase HPLC and ESI-MS, indicated that the cyclic *N*-acyl hydrazone dimers were the dominant products (>95% yield) from the deprotection of the *meta* substituted dimethyl acetal functionalised hydrazides (**6**, **9**, **10**, **11**, **14**, **15** and **16**). Only trace amounts of higher macrocycles, trimer and tetramer, could be detected by ESI-MS. Increasing the concentration of the dimethyl acetal functionalised steroidal hydrazide from 5 to 20 mM did not affect

this distribution to any significant extent, suggesting that the shape of these monomers is such that they are strongly predisposed towards the formation of cyclic dimer.[25]

The reaction mixtures derived from the hydrazides **15** and **6** were neutralised with Et_3N and subjected to preparative thin layer chromatography. The pure *N*-acyl hydrazone macrocyclic dimers **26** and **27** (Fig. 2 and 3) were isolated in high yields (~90%), and were soluble in acid free $CHCl_3$ to a concentration of 20 mM.[26]

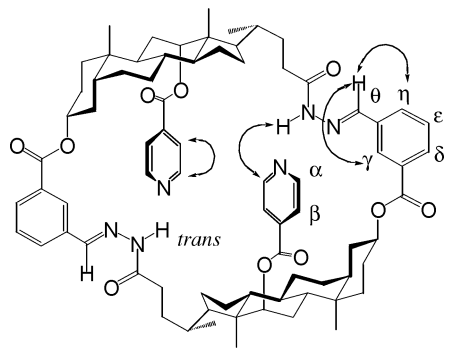


26

Fig. 2 Structure of cyclic dimer **26**.

The ^{1}H NMR spectrum of **26** (Fig. 2) in $CDCl_3$ was surprisingly simple, given that one might expect conformational or geometrical isomerism about the *N*-acyl hydrazones. That cyclisation had indeed taken place could be readily ascertained from the disappearance of the methoxy methyl groups of **15** at 3.34 ppm, the dimethyl acetal CH singlet at 5.43 ppm, the hydrazide NH resonance, at 6.84 ppm, and hydrazide NH_2 resonance, at 3.90 ppm, as well as the appearance of new signals in **26** due to the azamethine proton, Hμ at 7.55 ppm and the *N*-acyl hydrazone NH signal at 9.34 ppm. Other shifts, could also be observed in the aromatic and steroid regions of **26** compared with **15**. Particularly noticeable was the emergence and separation of the two diastereotopic 23-H protons from the forest of steroid peaks to 2.57 and 2.96 ppm.

N-Acyl hydrazones can exist in several possible arrangements and geometrical isomers: *cis E*, *cis Z*, *trans E* or *trans Z*.[27] The formation of rigid macrocycles, especially under equilibrating conditions, might be expected to favour the most stable geometry or arrangement; in this case probably an *E* arrangement about the *N*-acyl hydrazone NH. The NOESY spectrum of **26** in $CDCl_3$ at room temperature revealed strong nOe cross-peaks between the



27

Fig. 3 Structure of cyclic dimer **27**.

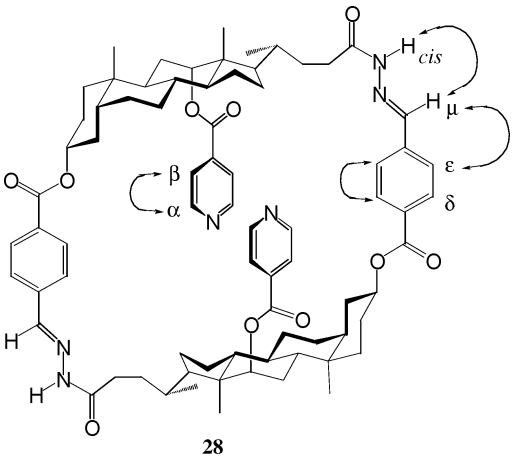
N-acyl hydrazone resonance, at 9.34 ppm, and the azamethine proton, Hμ, at 7.55 ppm. Further nOe cross-peaks could be observed between Hμ and Hη, at 8.08 ppm. This strongly supports a *cis E* geometry about the *N*-acyl hydrazone. That the hydrazone NH, is not directed into the cavity would seem to be supported by the lack of any nOe cross-peaks between the *N*-acyl hydrazone NH and the steroid 22-H or 23-H resonances.

While the ^{1}H NMR spectrum of **27** in $CDCl_3$ was also simple, it differed from that of **26** in two noticeable respects (Fig. 3). Thus, the *N*-acyl hydrazone NH resonance of **27** was observed at 10.85 ppm, compared to 9.34 ppm in **26**, and the azamethine proton, Hθ in **27** appeared at 8.62 ppm, compared to 7.55 ppm in **26**. The fact that the presence of the pyridine-4-carboxylate at the 12-position of the steroid could stabilise an otherwise disfavoured conformation was intriguing: the NOESY spectrum revealed no nOe cross-peak between the *N*-acyl hydrazone resonance at 10.85 ppm and the azamethine proton, Hθ, at 8.62 ppm, but a cross-peak was observed between Hθ and Hη, at 7.87 ppm and also between the *N*-acyl hydrazone NH and the 23-H steroid resonances, suggesting the presence of a *trans E N*-acyl hydrazone geometry. A weak nOe cross-peak could also be observed between the *N*-acyl hydrazone NH and the α-pyridyl proton of the pyridine substituent, at 8.65 ppm. This would seem to confirm that the pyridine substituent stabilises the *trans E N*-acyl hydrazone conformation. The NOESY spectrum also contains exchange peaks, indicating an alternative conformation populated to a lesser extent. A CPK model of **27** suggests that a pair of intramolecular hydrogen bonds, between the pyridine substituents and *N*-acyl hydrazone *trans* NH could account for the stabilisation of this conformation, hence the downfield shift ($\Delta\delta = -1.5$ ppm) of this proton, compared to the *N*-acyl hydrazone *cis* NH in **26**.

In order for a template to perturb the equilibrium of a DCL, a range of host structures must be present for templates to distinguish between. DCLs containing **26** and **27** do not satisfy this requirement due to their low diversity: distribution of macrocycles was needed. A relatively simple way to change the geometry of the monomers was to switch the aldehyde from *meta* to *para*.

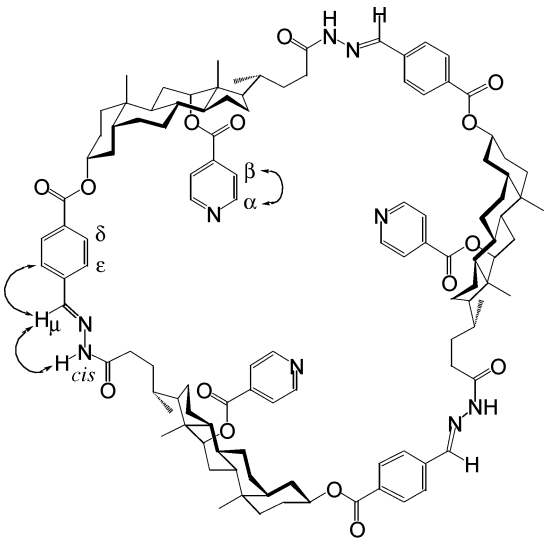
When a 5 mM solution of the hydrazide building block (**12**) in CH_2Cl_2 was deprotected using 5% TFA v/v at room temperature, direct analysis of the reaction mixture by HPLC and ESI-MS revealed the presence of the cyclic dimer (**28**, Fig. 4) and the cyclic trimer (**29**, Fig. 5), in a 3 : 1 ratio after equilibrium was reached, typically in 4–6 h. No **12** or linear, uncyclised species were observed. Increasing the concentration of **12** led to increasing amounts of trimer and above 50 mM some cyclic tetramer, as expected.[28] This was an encouraging result and interesting because the overall flexibility of the molecule had not been changed, in the sense of increasing the number of rotatable bonds, merely the direction in space of the aldehyde. The cyclic dimer (**28**) and the cyclic trimer (**29**) were readily separated by preparative thin layer chromatography to afford reasonable quantities of the pure macrocycles.

The ^{1}H NMR spectrum of the cyclic dimer (**28**, Fig. 4) in $CDCl_3$ was quite different from that of its regioisomer (**27**, Fig. 3). As well as the usual downfield shifts of the 23-H steroid protons in **28** compared to **12**, a considerable upfield shift ($\Delta\delta = -0.54$ ppm) of the α-pyridyl and β-pyridyl ($\Delta\delta = -0.20$ ppm) protons of **28** compared to **12** as well as the Hδ,ε proton was observed. This should be compared with an upfield shift of $\Delta\delta = -0.17$ ppm for



28

Fig. 4 Structure of cyclic dimer **28**.



29

Fig. 5 Structure of cyclic trimer **29**.

the α-pyridyl protons and $\Delta\delta = 0$ ppm for β-pyridyl protons of **27** compared to the hydrazide **6** and may be indicative of π–π stacking of the two pyridine substituents in **28**; this is less favourable in **27**, due to the *meta* substituted aldehyde substituent at the 3-position. The lowfield azamethine and *N*-acyl hydrazone resonances of **28** compared to **27**, suggest the *N*-acyl hydrazone adopts a *cis E* geometry in **28**.

The arrangement of the cyclic hydrazone was confirmed by the NOESY spectrum of **28** in $CDCl_3$ at room temperature. A strong nOe cross-peak was observed between the *N*-acyl hydrazone NH, at 9.17 ppm, and the azamethine proton, Hμ, at 7.64 ppm. Also an nOe cross-peak was observed between the azamethine proton, Hμ, and Hε, at 7.49 ppm. That the *N*-acyl hydrazone NH was not directed into the cavity was deduced from the lack of any significant nOe cross-peak between the *N*-acyl hydrazone NH and the 23-H steroid protons. The change of geometry from *meta* (**27**) to *para* (**28**) at the 3-position renders the *trans E N*-acyl hydrazone geometry unfavourable compared to the *cis E* geometry. The solution phase structure was also found in the solid state. An X-ray crystal structure of **28**, obtained by slow evaporation of a solution of **28** in CD_2Cl_2, revealed the geometry of the *N*-acyl hydrazone to be *cis E*.[16]

The ^{1}H NMR spectrum of the macrocyclic *N*-acyl hydrazone trimer (**29**, Fig. 5) also derived from the hydrazide **12**, differs from that of the cyclic dimer (**28**) in all of the key resonances. The azamethine proton, Hμ at 7.86 ppm and the *N*-acyl hydrazone NH are downfield shifted compared with **12**. In this case the NH proton is extremely downfield shifted to 11.10 ppm. Also noticeable was the absence of the large upfield shift in the α and β-pyridyl protons in **29**, $\Delta\delta = +0.09$ ppm and $\Delta\delta = +0.05$ ppm respectively, compared to **28**. While the downfield shift of the *N*-acyl hydrazone NH suggests the presence of hydrogen-bonding, COSY and NOESY experiments on a solution of the cyclic trimer (**29**) in $CDCl_3$ support the presence of a *cis E* geometry. Thus, an nOe cross-peak was observed between the *N*-acyl hydrazone NH, at 11.10 ppm and the azamething proton, Hμ, at 7.64 ppm. Furthermore an nOe cross-peak was observed between Hμ and Hε, at 7.70 ppm. The reason for the downfield shift of the *N*-acyl hydrazone NH was not clear, but it may be due to intermolecular hydrogen-bonding between two, or more, different trimer units.[29]

If it were true that simply changing the geometry of the aldehyde at the 3-position from *meta* to *para* were responsible for increasing the stability of the cyclic trimer compared to the cyclic dimer one might expect that deprotection of the *para* substituted monomer **18** would result in a similar product distribution to that obtained from the deprotection of **12**. This was not found, however, to be the case: deprotection of a 5 mM solution of **18** in CH_2Cl_2 led initially to a large distribution of cyclic products (dimer, trimer and tetramer), by HPLC, but over a period of several hours the amounts of trimer and tetramer decreased. At equilibrium the ratio of dimer to trimer was found to be 8 : 1, with no significant amounts of the tetramer. The dimer and trimer were separated by preparative chromatography, allowing ESI-MS analysis and confirmation of the HPLC assignments. The ^{1}H NMR spectra of the cyclic dimer and trimer could be recorded, but their poor solubility in $CDCl_3$ required the addition of d_4-MeOH, depriving us of the valuable structural information supplied by the *N*-acyl hydrazone NH signal. Increasing the concentration of **18**, from 5 mM to 10 mM, led to a cyclic dimer to trimer ratio of 6 : 1.

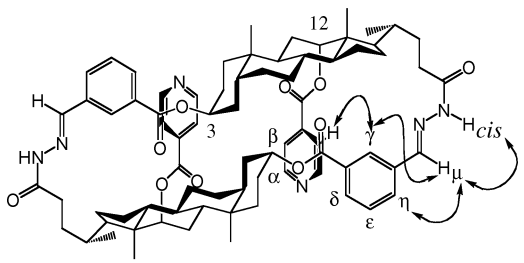
The results of deprotecting the *para* substituted dimethyl acetal functionalised hydrazides, **17** and **19**, seem to confirm that the presence of pyridine-4-carboxylate substituent at the 12-position of the steroid controls the distribution of the macrocycles formed on deprotection and, in addition, increases the solubility of the macrocycles in $CDCl_3$. Thus, deprotection of a 5 mM solution of **17** in CH_2Cl_2, using the standard conditions of 5% TFA v/v led to a single macrocyclic product, the dimer, by HPLC and ESI-MS. The solubility of the neutral macrocycle, derived from **17**, in $CDCl_3$ was initially good, but after several minutes a precipitate formed, leading to broad signals in the ^{1}H NMR spectrum.

The *N*-acyl hydrazones derived from **19** were designed to afford a platform for templating experiments with polyhydroxylated molecules in $CHCl_3$. However, addition of TFA to a 5 mM solution of **19** in CH_2Cl_2 and $CHCl_3$, led to the formation of a precipitate after an hour. Filtration of the precipitate and analysis of the filtrate revealed the presence of only a single macrocycle, which does not satisfy the diversity requirements for DCLs. Carrying out the deprotection in DMSO did not lead to the formation of a

precipitate, however, still yielded only a single macrocyclic product (dimer).

In an attempt to understand the effect of flexibility on product distribution further the *meta* substituted glycine extended dimethyl acetal functionalised hydrazides (**21** and **23**) were studied. However, deprotecting **21** and **23** at a concentration of 5 mM in CH_2Cl_2 again led to the cyclic dimer as major product (>90%), with cyclic trimer making up the remainder of the product. Increasing the concentration of **21** and **23** to 20 mM did not increase the diversity of reaction products significantly.

The final modification to the steroid structure, that is, inversion at the 3-position of the steroid to form the *meta* substituted dimethyl acetal functionalised hydrazide (**25**), was examined in an attempt to increase the diversity of macrocycles at equilibrium. In the initial distribution of products identified by HPLC and ESI-MS a range of macrocyclic *N*-acyl hydrazones from cyclic dimer to pentamer were observed. This, however, proved to be only an ephemeral, kinetic, distribution and after 24 h the distribution of products collapsed leaving the cyclic *N*-acyl hydrazone dimer (**30**) as the only detectable product at equilibrium (Fig. 6).



30

Fig. 6 Structure of cyclic dimer **30**.

The ^{1}H NMR spectrum of **30** (obtained by preparative thin layer chromatography) was, as expected, quite different from its stereoisomer (**27**) in that the *N*-acyl hydrazone NH resonance appears at 9.58 ppm in **30** and the azamethine proton at 7.79 ppm, compared with 10.85 ppm and 8.62 ppm for the same resonances in **27**. The *cis E* geometry of **30** was confirmed by NOESY: a strong nOe cross peak was observed between the *N*-acyl hydrazone NH resonance at 9.58 ppm and the azamethine proton at 7.79 ppm. In addition a strong nOe cross-peak could be observed between the azamethine proton and Hη, at 7.50 ppm. The most dramatic difference between **27** and **30** is the large upfield shift of Hγ ($\Delta\delta$ = +0.84 ppm) in **30**, attributed to the close proximity of the 12-pyridine substituent in the constrained macrocycle. There does not appear to be any evidence of π–π stacking between the two 12-pyridine substituents in **30**.

The range of structural modifications made to the basic bile acid core has taught us about the factors that influence the distribution of macrocyclic *N*-acyl hydrazones at equilibrium. Thus, a *meta* substituted aldehyde leads to predominant formation of macrocyclic *N*-acyl hydrazone dimers at equilibrium, regardless of the stereochemistry at the 3-positon and the presence of a glycine extension. A *para* substituted aldehyde on the other hand enables significant formation of macrocyclic trimer, with the caveat that a pyridine substituent must be present at the 12-position. This also has the benefit of increasing the solubility of the macrocycle in CH_2Cl_2 and $CHCl_3$. If we wish to use these macrocycles for the purposes of templating in chlorinated solvents we must therefore address these design issues and incorporate these lessons into future molecules.

Demonstration of reversibility and exchange experiments

In order to demonstrate that the distribution of macrocycles is at equilibrium it was necessary to show that the formation of macrocyclic steroidal *N*-acyl hydrazones is reversible. There are two relatively simple experiments that accomplish this. The most satisfying is to take a library solution, isolate one of the macrocycles and show that this, under the reaction conditions, gives rise to the original library distribution. Indeed, when a 2.5 mM solution of pure **28** in CH_2Cl_2 was treated with 5% TFA v/v. HPLC and ESI-MS indicated the presence of *both* **28** and **29** in a 3 : 1 ratio after 8 h. When this experiment was repeated using a 1.7 mM solution of pure **29** in CH_2Cl_2 a 3 : 1 ratio of **28** and **29** was observed by HPLC and ESI-MS after 6 h (Fig. 7). This confirms both the reversibility of steroidal macrocyclic *N*-acyl hydrazone formation and that the product distribution is under thermodynamic control.

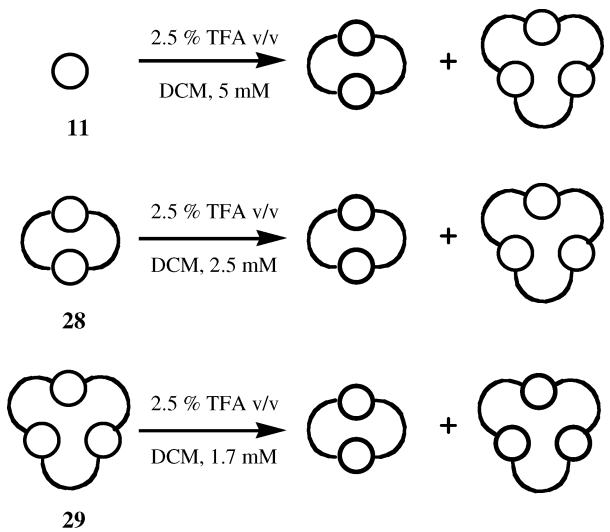


Fig. 7 Demonstration of thermodynamic control.

Alternatively, reversibility can be demonstrated by an exchange experiment using two macrocycles that may be distinguished analytically. If the formation of two macrocyclic dimers is reversible they should give rise to a statistical mixture of homo and heterodimers in the presence of acid. In order to test this, a series of experiments was carried out using several dimethyl acetal functionalised hydrazides. The first experiment involved mixing the preformed macrocycles and the second experiment involved deprotecting the two dimethyl acetal functionalised hydrazide monomers *in situ*. These experiments were compared to see, if any, differences between kinetic and thermodynamic distributions of products.

We were particularly interested to see if the kinetic stability of macrocyclic *N*-acyl hydrazone dimers would prohibit ring opening and exchange, as might, perhaps, be expected for such favoured structures. If kinetic stability were the underlying cause then one might expect to see no mixing of preformed cyclic dimers.

When the monomers are deprotected *in situ*, however, one would expect to see the heterodimer and for this to be a stable entity. With monomers **6** and **14** the results of the two mixing experiments were surprising. No mixing of the preformed macrocyclic *N*-acyl hydrazones derived from **6** and **14** was observed by HPLC or ESI-MS after a period of seven days. This was taken as evidence of the kinetic stability of the dimers. When **6** and **14** were deprotected together under the same conditions, the mixed dimer could be observed by HPLC and ESI-MS, but over a period of 24 h it disappeared (Fig. 8). This surprising example of self-sorting prompted a further series of experiments using other macrocyclic *N*-acyl hydrazone dimers.

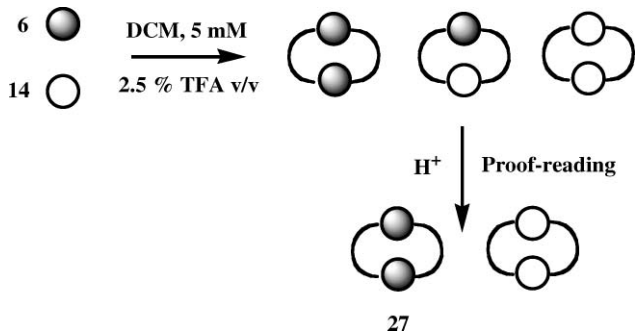


Fig. 8 Proof-reading in DCLs.

When equimolar 5 mM solutions of the two macrocyclic *N*-acyl hydrazone dimers derived from **9** and **14**, were mixed at room temperature in the presence of 5% TFA v/v the formation of heterodimer could be observed by both HPLC and ESI-MS over a period of seven days. The slow nature of the reaction was, in part, to be expected due to the constrained nature of the macrocycles and the bias towards ring closure over a second ring opening. Confirmation of this result was obtained by deprotecting **9** and **14** together at the same concentration. A statistical mixture of the three possible products was observed and, significantly, did not change over a period of seven days (Fig. 9). Further experiments using the monomers **14** and **16** also indicated that exchange of macrocyclic *N*-acyl hydrazones is a feasible process, occurring over a period of several days. Mixing of **10** and **14** did not lead to self-sorting.

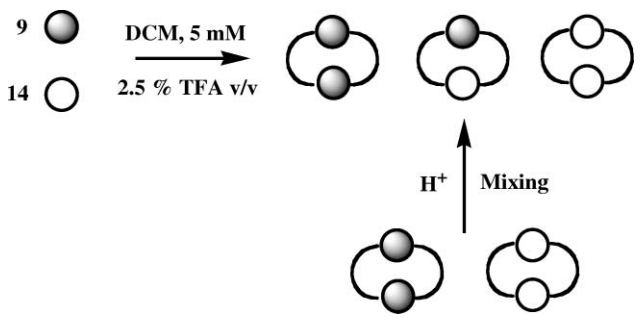


Fig. 9 Demonstration of thermodynamic control, without proof-reading.

This behaviour was unexpected. There does not appear to be any obvious reason why two steroids which both form macrocyclic dimers should not mix together to form a heterodimer. On the other hand there are minor differences in the conformations of the cyclic dimer **27** and the macrocyclic dimer derived from **14** as indicated by their ^{1}H NMR spectra. These geometrical differences could be responsible for the slight differences of the monomers, thus disfavouring the heterodimer as compared to the homodimers. This reasoning is limited, however, to a consideration of solutions of the neutral macrocycles and the exchange behaviour is observed in the presence of TFA. One can envisage both kinetic and thermodynamic grounds for the differentially substituted monomers refusing to mix, but evidence in favour of either was not available from these experiments.

A further example of self-sorting could be observed by mixing the monomers **14** and **25**. Upon addition of TFA (5% v/v) to a 2.5 mM solution of **14** and **25** in CH_2Cl_2, analysis by HPLC and ESI-MS indicated the formation of several mixed products as well as the higher oligomers of monomer **25**. Over a period of 24 h, however, these mixed and higher macrocycles were proof-read, to leave just the two homodimers, derived from **14** and **25**, by HPLC and ESI-MS. The phenomenon of self-sorting seems to be connected with the presence of a 4-pyridine substituent in the 12-position of the steroid.

Conclusion

We have synthesised a series of steroid based building blocks for hydrazone based dynamic combinatorial chemistry containing a hydrazide and a dimethylacetal protected aldehyde. Upon treatment of a building block in organic solvents with TFA the dominant products were cyclic dimers at thermodynamic equilibrium. Larger macrocycles were present as kinetic intermediates, and macrocycles with different conformations tend to self-sort rather than mix. The use of these macrocycles for the purpose of templating in chlorinated solvents rely on careful design of the building blocks. It may be important to avoid self-templating motifs in the building blocks, however, this is an issue that can be difficult to predict.

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