Generating rapamycin analogues by directed biosynthesis: starter acid substrate specificity of mono-substituted cyclohexane carboxylic acids†

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We report a convenient synthesis of 4-fluorocyclohexanoic acid, and an insight into the rules governing acceptance of starter acid analogues in the precursor-directed biosynthesis of rapamycin.

Rapamycin is a potent immunosuppressant and also exhibits antiproliferative and antitumour activity.[1,2] The polyketide macrolide was first isolated from a culture of *Streptomyces hygroscopicus* NRRL 5491, obtained from a soil sample collected on Easter Island (Rapa Nui).[3]

Due to its low level of nephrotoxicity,[4] rapamycin is commonly used for the treatment of patients undergoing renal transplantation. However, due to rapamycin's poor physiochemical properties there is continued interest in developing novel analogues with improved pharmacological properties. A handful of total syntheses of this challenging molecule have been reported.[5] The complexity and length of these syntheses preclude their use for lead optimisation, and other strategies of accessing analogues have been employed. Modification of the macrolide, by chemical and enzymatic methods,[6] and more recently through exploitation of the natural biosynthetic pathway has been possible.[7,8]

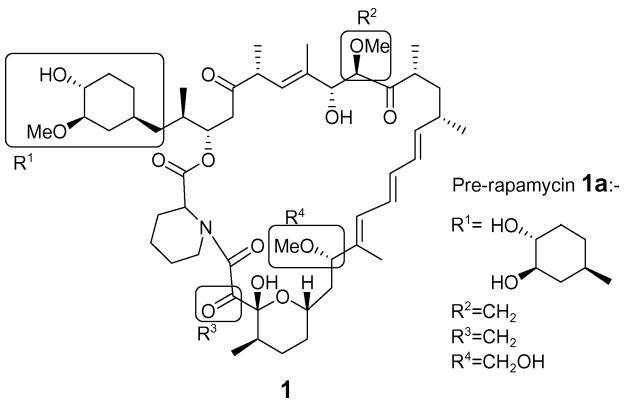

Fig. 1 Rapamycin **1** and pre-rapamycin **1a**.

Rapamycin is biosynthesised by a modular polyketide synthase (PKS), that utilises (1*R*,4*R*,5*R*)-4,5-dihydroxycyclohex-1-ene carboxylic acid as a starter acid.[9] It has previously been shown that cyclohexane and cycloheptane carboxylic acid analogues of the starter acid can be accepted by the polyketide synthase in competition with the natural starter acid to produce a mixture of rapamycins in a production culture.[7] Notably, cyclohexane and cycloheptane rings of rapamycins resulting from incorporation of these starter acids are mono hydroxylated. Gregory *et al.* removed genes *rapKIJMNOQL* from the rapamycin biosynthetic locus to generate *S. hygroscopicus* (MG2-10), a mutant disrupted in the biosynthesis, or regulation of the biosynthesis, of the starter acid as well as the post-PKS modification of the macrolide. The mutant is able to produce pre-rapamycin **1a** when supplemented with (1*R**,3*R**,4*R**)-3,4-dihydroxycyclohexane carboxylic acid **2** or if the gene *rapK* is re-introduced.[10] Supplementation of this mutant with cyclohexane carboxylic acid **3** results in the formation of a rapamycin analogue bearing a 4-hydroxycyclohexane moiety, whereas supplementation with cyclohex-1-ene carboxylic acid **4** leads to the generation of a rapamycin analogue bearing a 3-hydroxycyclohexane moiety (Table 1).[11] Crucially when supplemented with several commercially available starter acid analogues, the equivalent pre-rapamycin analogue is produced in the absence of pre-rapamycin itself.[11] This is an ideal system to interrogate both the apparent requirement for hydroxylation of the starter acid prior to incorporation into the polyketide, and the breadth of substrate specificity of the PKS in general. In order to determine whether hydroxylation is indeed required we set out to synthesise and feed a range of hydroxylated starter acid analogues, and sterically similar starter acid analogues that could not be hydroxylated due to the presence of electronegative fluorine substituents.

4-, 3- And 2-hydroxycyclohexane carboxylic acids **5**, **6** and **7** were generated as a mixture of *cis* and *trans* isomers through catalytic hydrogenation of their corresponding hydroxybenzoate ethyl esters,[12] followed by base catalysed hydrolysis. The resultant hydroxylated cyclohexane carboxylic acids were administered at a final concentration of 2 mM, as previously described[11] to cultures of *S. hygroscopicus* (MG2-10) and the production of pre-rapamycin analogues analysed by LC-MS (Table 1). Incorporation of 4-hydroxycyclohexane carboxylic acid **5** results in the production of highest levels of equivalent pre-rapamycin (33 mg L^{-1}). This was anticipated as the administration of cyclohexane carboxylic acid **3** results in the generation of the same pre-rapamycin analogue in which the cyclohexane ring is hydroxylated at C-4. The titer of the pre-rapamycin analogue resulting from the incorporation of 3-hydroxycyclohexane carboxylic acid **6** was considerably lower (3 mg L^{-1}). The pre-rapamycin analogue generated is the same as that observed when cyclohex-1-ene carboxylic acid **4** is administered to the mutant. No pre-rapamycin analogue could be identified when 2-hydroxycyclohexane carboxylic acid **7** was fed.

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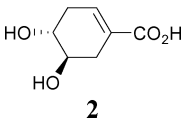
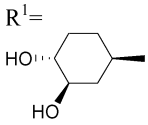
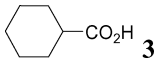
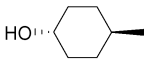
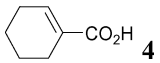
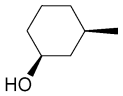
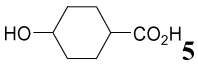
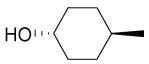
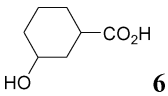
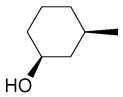
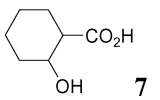
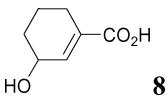
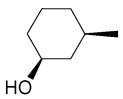
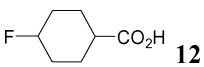
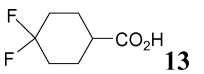
† Electronic supplementary information (ESI) available: Full details of the synthesis of fluorocyclohexane carboxylic acid and LC-MS data for the rapamycin analogues generated. See DOI: 10.1039/b614519c

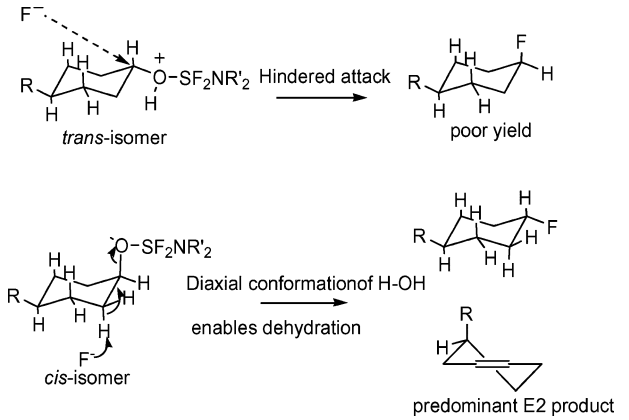
Table 1 Incorporation of synthetic starter acids into pre-rapamycin

Starter acid analogue	Pre-rapamycin analogue (Fig. 1)	Pre-rapamycin analogue produced/mg L^{-1}
 **2**	R^1=  R^2=CH_2, R^3= CH_2	Yield not reported[11]
 **3**	R^1=  R^2=CH_2, R^3= CH_2	100[11]
 **4**	R^1=  R^2=CH_2, R^3= CH_2	22[11]
 **5**	R^1=  R^2=CH_2, R^3= CH_2	33 ± 3
 **6**	R^1=  R^2=CH_2, R^3= CH_2	3 ± 1
 **7**	—	0
 **8**	R^1=  R^2=CH_2, R^3= CH_2	18 ± 3
 **12**	—	0
 **13**	—	0

The incorporation of cyclohex-1-ene carboxylic acid **4** and its conversion to a 3-hydroxycyclohexane rapamycin prompted us to synthesise 3-hydroxycyclohex-1-ene carboxylic acid **8**[13] for use in feeding experiments. This compound was incorporated to give a significantly higher titer of pre-rapamycin analogue (18 mg L^{-1}) than its saturated analogue **6** (3 mg L^{-1}). This is probably a reflection on its greater similarity to the unsaturated natural starter acid 4,5-dihydroxycyclohex-1-ene carboxylate.[9]

A carbon–fluorine bond is almost isosteric with the carbon–oxygen bond of a hydroxyl group (1.38 Å *versus* 1.42 Å respectively).[14] It may therefore be postulated that if high levels of incorporation of 4-hydroxycyclohexane carboxylic acid is due to steric recognition of the starter acid analogue alone, then incorporation of 4-fluorocyclohexane carboxylic acid should result. The presence of an electron-withdrawing fluorine, at this position, is also likely to preclude further oxidation of the ring. Fluorinated cyclohexane carboxylic acids are important synthetic precursors for materials and medicinal chemistry.[15,16] Despite this usefulness, and apparent structural simplicity, these compounds are difficult to synthesise. The only literature synthesis of **12** reports a disappointing yield of 6.7% for the fluorination step.[16]

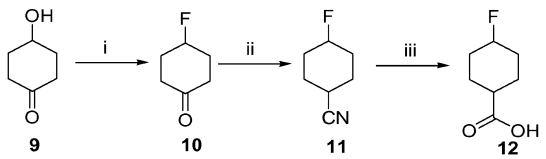
Also it has been shown that, when compared to other secondary alcohols, cyclohexanol is difficult to fluorinate and that monosubstituted cyclohexanols represent an even greater challenge.[17] Due to the steric crowding in such systems and the basicity of the fluorine nucleophile, attempted fluorinations of these compounds often lead to the formation of the unwanted dehydration product instead (Scheme 1). Attempts to access the fluorinated cyclohexane



Scheme 1 Explanation for poor yields in the fluorination of substituted cyclohexanols, adapted from Mange and Middleton.[17]

carboxylic acids by catalytic hydrogenation of the corresponding fluorobenzoates were not successful, resulting in inseparable mixtures of compounds of which > 90% did not retain the fluorine substituent.[18]

Having attempted modifications of the two approaches described above with little improvement to yield, we explored the fluorination of 4-hydroxycyclohexanone **9** (Scheme 2), the rationale being that the hydroxyl would be less sterically hindered and thus more accessible to nucleophilic attack by fluoride. Yields for the fluorination step of 18% and 24% were achieved using diethyl amino sulphur trifluoride (DAST) and HF–pyridine respectively. This represents a significant (3.5 fold) improvement on the analogous reported yield of only 6.7% for the fluorination of a 4-hydroxycyclohexane carboxylate derivitive.[16] 4-Fluorocyclohexanone **10** was then converted, in two steps, to 4-fluorocyclohexane carboxylic acid **12** (Scheme 2).



Scheme 2 Procedure for the generation of 4-fluorocyclohexane carboxylic acid. *Reagents and conditions*: (i) HF–pyridine, 0 °C, 24%; (ii) TosMIC, ^{t}BuOH, ^{t}BuOK, DME, RT, 64%; (iii) NaOH (1 M), 80 °C, 65%.

4-Fluorocyclohexanoic acid **12** and a commercial sample of 4,4-difluorocyclohexanoic acid **13**‡ were administered to the test organism described above. No pre-rapamycins were generated indicating the necessity for hydroxylation of the ring of the starter acid prior to its incorporation and showing that fluorination precluded further enzymatic oxidation of the substrates *in vivo.*

In conclusion we describe a considerably improved synthesis of 4-fluorocyclohexane carboxylic acid (**12**), with a 3.5 fold improvement in the fluorination step. The administration of both 4- and 3-hydroxycyclohexane carboxylic acid and 3-hydroxycyclohex-1-ene carboxylic acid to *S. hygroscopicus* (MG2-10) lead to the production of pre-rapamycin analogues whereas 2-hydroxy, 4-fluoro- and 4,4-difluorocyclohexane carboxylic acid does not. Although a carbon–fluorine bond is almost isosteric with a carbon–oxygen bond this is not sufficient for recognition and acceptance by the PKS; this is likely to be due to the substantially different electronic and hydrogen bonding properties. Interestingly, the low level incorporation of tetrahydro-2*H*-pyran-4-carboxylic acid into a pre-rapamycin analogue by *S. hygroscopicus* (MG2-10) is reported without hydroxylation of the ring.[11] This observation further supports the hypothesis that the ring must carry a hydrogen bond acceptor, prefereably a hydroxyl moiety at the 4-position, in order for starter acid incorporation by the PKS. Furthermore, the presence of fluorine at C-3 or C-4 is likely to prevent enzymatic oxidation of the cyclohexyl ring. This information is valuable as cyclohexanoic acid fluorohydrins are accepted as substrates by the PKS (although the material was uncharacterized in terms of regio- and stereochemistry).[19] Further experiments are in progress to investigate further the regio- and stereochemical requirements of cyclohexane ring substitution for PKS starter unit recognition.

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Notes and references

‡ Ethyl 4,4-difluorocyclohexane carboxylate was purchased from Matrix Scientific and fed as both the ethyl ester and the free acid.

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