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An Expeditious Route to Fluorinated Rapamycin Analogues by Utilising Mutasynthesis

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Rapamycin is a drug with several important clinical uses. Its complex structure means that total synthesis of this natural product and its analogues is demanding and lengthy. A more expeditious approach is to utilise biosynthesis to enable the generation of otherwise synthetically intractable analogues. In order to achieve this, rules governing biosynthetic precursor substrate preference must be established. Through determining these rules and synthesising and administering suitable substrate precursors, we demonstrate the first generation of fluorinated rapamycin analogues. Here we report the generation of six new fluororapamycins.

Introduction

Rapamycin is a polyketide natural product that selectively inhibits the kinase mammalian target of rapamycin (mTOR), a central regulator of the cell cycle and proliferation.[1] Inhibition of mTOR has significant pharmacological value. This fact is underscored by rapamycin's clinical application as an immunosuppressant therapy (Rapamune®) following organ transplantation, and the recent discovery that inhibition of mTOR signalling by rapamycin extends lifespan in genetically heterogeneous mice, even when administered late in life.[2] Recently the semisynthetic analogues temsirolimus (Torisel®) and everolimus (Afinitor®) were approved as mTOR inhibitors for the treatment of advanced renal cell carcinoma, and further analogues are in late stage clinical trials.[3] These semisynthetic analogues are similarly modified at the C40 secondary hydroxyl group on the highly substituted cyclohexane moiety, attesting to the limitations of this approach. Moreover, although a number of total syntheses of this challenging molecule and its analogues have been described,[4] the complexity and length of these syntheses preclude their utility in lead optimisation.

An alternative and expeditious route through which to generate new rapamycin analogues (rapalogues) is the exploitation of its biosynthetic pathway. Mutasynthetic approaches have previously been successfully employed to generate analogues of a number of natural products.[5] Notably, the aforementioned cyclohexane moiety of rapamycin can be targeted through the application of precursor-directed feeding and mutasynthesis methods.[6,7] For example, administration of suitable cyclohexanecarboxylic and cycloheptanecarboxylic acids to a mutant (MG2-10, assigned BIOT-3016) of the producing organism, *Streptomyces hygroscopicus*, which cannot produce the natural starter acid required to initiate polyketide synthesis by the polyketide synthase (PKS), enables the selective production of prerapamycin analogues.[7] Prerapamycin (**1b**, Scheme 2, below) is the untailored polyketide that lacks all post-polyketide synthase modifications, that is, oxidation at C9 and C27, and O-methylation at C16, C27 and C39. Studies with the prerapamycins have demonstrated a lack of antiproliferative activity against cancer cell lines and in the mixed lymphocyte reaction (a measure of immunosuppressive activity) as reported in the Supporting Information. The advantage of using this strain background as a model system to access substrate specificity is that a single rapalogue is produced if a suitable starter unit is fed. Rules governing the incorporation of the starter unit may be readily determined by using this model system and then applied to the production of more advanced compounds.

The presence of a hydrogen-bond acceptor on the PKS starter acid has been shown to be crucial for the incorporation of the cyclohexanecarboxylic acid into rapamycin.[6–8] Exogenously added cyclohexane-1-carboxylic acid and cyclohex-1-enecarboxylic acid are utilised by the PKS to initiate biosynthesis only after they are first hydroxylated at the 4- and 3-positions, respectively.

The presence of fluorine in a molecule can confer beneficial physical and medicinal properties such as increased stability and improved bioavailability.[9] Although a C–F bond is almost the same length as a C–OH bond, the corresponding cyclohexanecarboxylic acids fluorinated at the 4-, 3- and 2-positions were not incorporated. This reinforces the idea that a hydrogen bond acceptor is required in order for the rapamycin PKS to utilise a carboxylic acid to initiate biosynthesis. In light of this we decided to investigate the ability of the PKS to utilise substrates with both a fluorine atom and a hydroxyl group,

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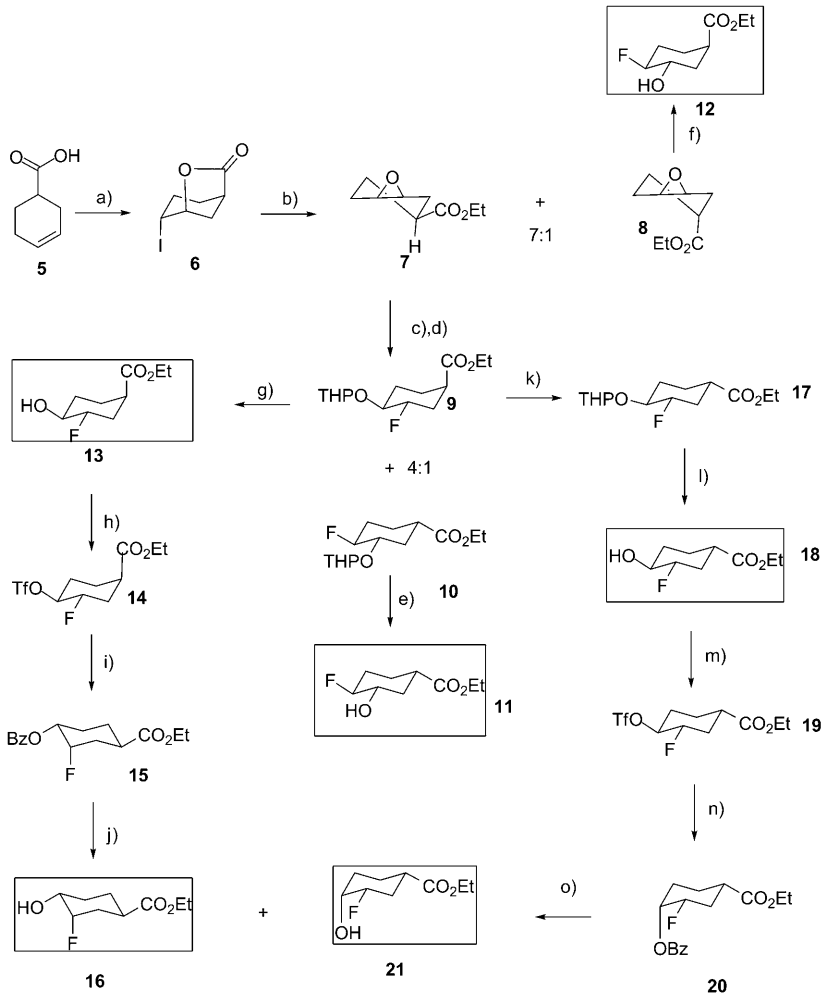


Scheme 1. Synthesis of ethyl (1*S**,3*R**,4*R**)-, (1*S**,3*R**,4*S**)-, (1*R**,3*R**,4*R**)- and (1*R**,3*R**,4*S**)-3-fluoro-4-hydroxy cyclohexanecarboxylate **13**, **16**, **18**, **21** and (1*R**,3*R**,4*R**)- and (1*R**,3*S**,4*S**)- 4-fluoro-3-hydroxycyclohexanoates **11** and **12** (boxed). a) I_2, KI, $NaHCO_3$, H_2O, RT, 16 h, 96%; b) EtONa, EtOH, THF, 0 °C, 16 h, 48% **7**, 7% **8**; c) Et_3N:HF, RT, 16 h, 76% as a mixture of **11** and **13**; d) DHP, H_2NSO_3H, 4 d, 52% **9**, 15% **10**; e) *p*-TsOH, EtOH, RT, 2 h, 76%; f) Et_3N:HF, RT, 76%; g) *p*-TsOH, EtOH, RT, 2 h, 99%; h) triflic anhydride, pyridine, DCM, 0 °C, 2 h, 76%; i) $(Bu)_4N^+BzO^-$, toluene, RT, 16 h, 65%; j) Na, EtOH, RT, 16 h, 57% **16**, 33% **21**; k) *t*BuOK , toluene, reflux, 40 min, 53%; l) *p*-TsOH, MeOH, RT, 2 h, 92%; m) triflic anhydride, pyridine, DCM, 0 °C, 2 h, 67%; n) $(Bu)_4N^+BzO^-$, toluene, RT, 16 h, 61%; o) Na, EtOH, RT, 16 h, 88% **21**, 4% **16**.

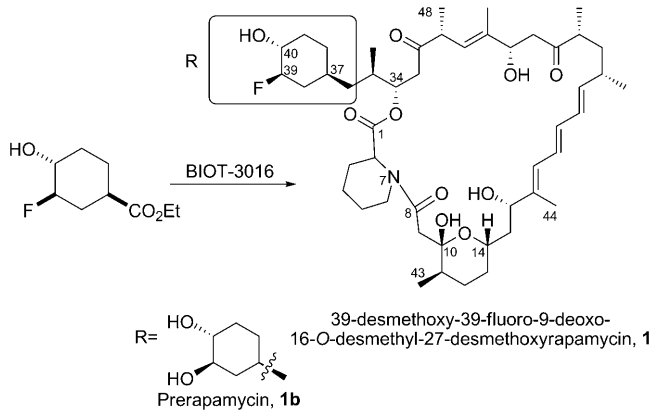
and thus we synthesised a series of appropriately substituted fluorohydrins (Scheme 1).

Results and Discussion

In order to determine the importance of relative stereochemistry of substituents on the cyclohexane ring for incorporation into the polyketide we synthesised the ethyl (1*S**,3*R**,4*R**)-, (1*S**,3*R**,4*S**)-, (1*R**,3*R**,4*R**)- and (1*R**,3*R**,4*S**)-3-fluoro-4-hydroxycyclohexanecarboxylates **13**, **16**, **18** and **21**, respectively. The issue of regiochemistry was also addressed by the synthesis and feeding of ethyl (1*R**,3*R**,4*R**)- and (1*R**,3*S**,4*S**)-4-fluoro-3-hydroxycyclohexanecarboxylates **11** and **12**.

In our general synthetic procedure (Scheme 1), relative stereochemistry was established through the iodolactonisation of cyclohex-3-ene carboxylic acid, followed by sodium ethoxide-mediated lactone opening.[10] The subsequent displacement of iodide by the released hydroxyl afforded the two epoxides, **7** and **8** in a 7:1 ratio, which were separated chromatographically and their relative stereochemistry determined from their NOESY spectra (Supporting Information). Synthesis of **11** and **13** was achieved by ring opening epoxide **7** with Et_3N:HF to yield ethyl (1*R**,3*R**,4*R**)-4-fluoro-3-hydroxycyclohexanecarboxylate **11** and ethyl (1*S**,3*R**,4*R**)-3-fluoro-4-hydroxycyclohexanecarboxylate **13** in a 4:1 ratio. In order to separate these regioisomers chromatographically, they were protected as their respective THP ethers, **9** and **10**. Following chromatographic separation, removal of the THP group under acidic conditions furnished compounds **11** and **13**. The fluorination of epoxide **8** gave fluorohydrin **12** as a single compound. In order to access ethyl (1*R**,3*R**,4*R**)-3-fluoro-4-hydroxycyclohexanecarboxylate **18**, the THP ether of ethyl (1*S**,3*R**,4*R**)-3-fluoro-4-hydroxycylclohexanecarboxylate **9** was epimerised by refluxing with *t*BuOK prior to deprotection of the THP ether. Ethyl (1*S**,3*R**,4*S**)-, and (1*R**,3*R**,4*S**)-3-fluoro-4-hydroxycyclohexanecarboxylate **16**, and **21** were accessed by inverting the hydroxyls of **13** and **18**, respectively. This was achieved by transforming the fluorohydrins **13** and **18** to their corresponding triflates followed by benzoylation using tetrabutylammonium benzoate to yield **15** and **20**. Hydrolysis of the benzoate esters under basic condition yielded the corresponding fluorohydrins **16** and **21** along with a small amount of epimerised product (Scheme 2). The relative stereochemistry of compounds **12**, **13**, and **18** was confirmed from the crystal structures of their PNB derivatives (Figure 1). Derivatives of **11**, **16** and **21** did not yield well to crystallisation; the relative stereochemistry of these compounds was clearly deduced from their NOESY spectra (Supporting Information).

Fluorohydroxycyclohexanecarboxylic acids and their ester derivatives were administered to *S. hygroscopicus* MG2-10 (BIOT-3016) at a final concentration of 2 mM. Using this strain, rapalogues are only generated if a suitable starter unit is administered as a carboxylic acid or carboxylic acid derivative.[7]



Scheme 2. Directing biosynthesis to generate 39-desmethoxy-39-fluoro-9-deoxo-16-*O*-desmethyl-27-desmethoxyrapamycin (**1**).

Previous attempts to generate fluorinated rapamycin analogues by administering 4-, 3- and 2-fluorocyclohexanecarboxylic acids to the strain had been unsuccessful, and we postulated that this was due to the requirement of a hydrogen-bond acceptor at the 3- or 4-position.[8] Hydroxylation of the fluorocyclohexane carboxylates by the strain was prevented by the presence of fluorine (cf. the facile hydroxylation of cyclohexanecarboxylic acid by this strain). When the six fluorohydrins **11**, **12**, **13**, **16**, **18** and **21** were administered to *S. hygroscopicus* MG2-10 (BIOT-3016), we observed the production of six new prerapamycin analogues (Table 1). Careful analysis by LC-MS/MS showed these were consistent with rapalogues carrying a fluorine atom in the cyclohexane moiety. Notably, the regiochemistry and relative stereochemistry of the fluorohydrins significantly affected the new rapalogue production titre. Our studies revealed a significant preference for location of the hydroxyl group at the 4-position, as suggested by previous studies.[6–8] For instance, the rapalogue resulting from administering **18** is six times more abundant than the equivalent rapalogue resulting from feeding **11**. Preference for a *trans* relationship between the hydroxyl and carboxylate groups was also evident, with the administration of **18** resulting in three times the titre of product compared to that resulting from administration of **13** to the cultures. With a 1,4-*trans* relationship in place, the presence of fluorine is well tolerated with little difference in preference for it occupying the axial or equatorial position.

In order to verify the identity of one new rapalogue, and thus validate our findings, we fed fluorohydrin **18** to a larger culture of *S. hygroscopicus* MG2-10 (BIOT-3016). From 1.5 L of fermentation broth we isolated 16.4 mg of the new compound **1** (Scheme 2) by using standard procedures as described previously.[6,7] The structure of **1** was determined by a combination of high-resolution MS and multidimensional NMR, (Supporting Information) with comparison to the structural data for closely related structures described previously.[5,6] As expected, **1** exists as two interconverting forms, and we assigned the signals corresponding to each form if possible. It is believed that these two forms correspond to isomers which result from *cis–trans* isomerism of the amide bond.

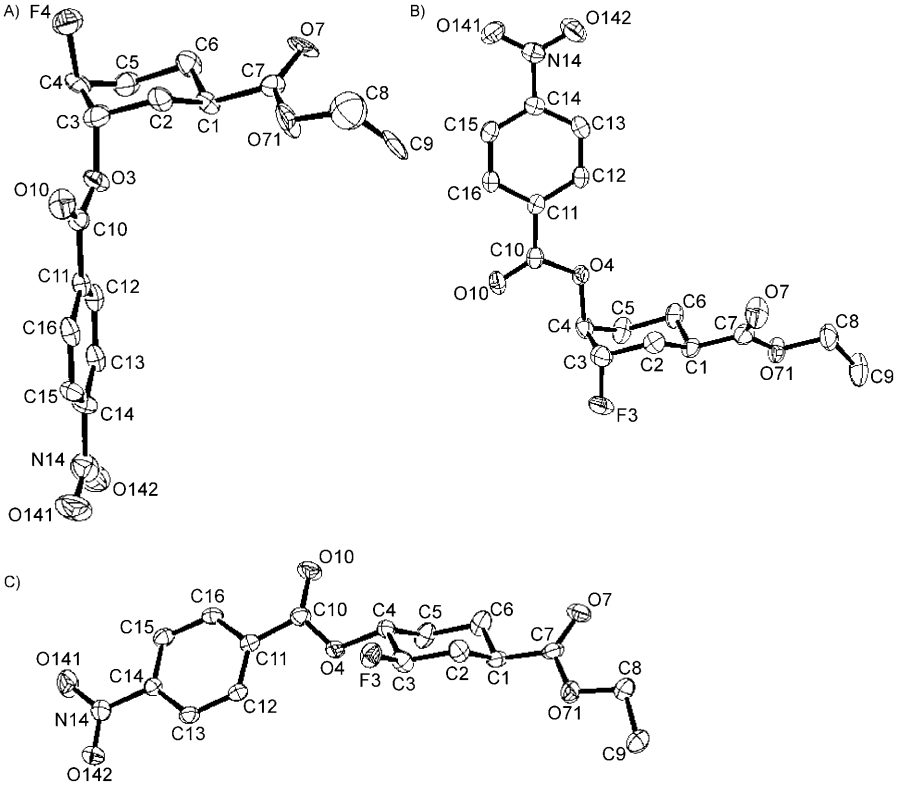
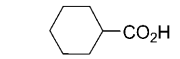
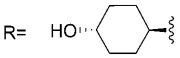
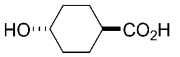
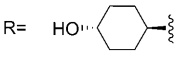
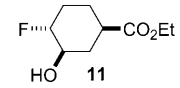
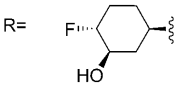
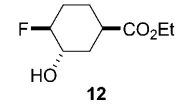
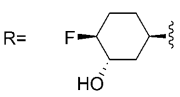
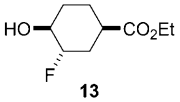
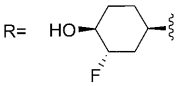
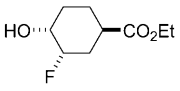
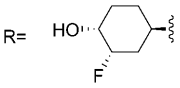
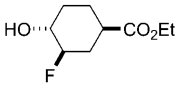
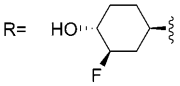
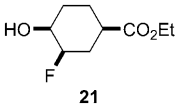
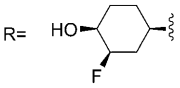


Figure 1. Crystal structures of A) **12**-PNB, showing the ethyl ester adopting the equatorial position and 4-fluoro and 3-*O*-PNB groups adopting the axial positions, B) **13**-PNB, showing the ethyl ester adopting the equatorial position and the 3-fluoro and 4-*O*-PNB groups adopting the axial positions, and C) **18**-PNB showing all groups equatorial. Hydrogen atoms have been omitted for clarity. Interestingly, a switch in conformation was observed for **12** and **13**, upon their conversion to the PNB derivative, placing the *O*-PNB group into the axial position. This change in conformation was not observed for **18**.

Conclusions

In summary, we report the generation of six fluorinated analogues of rapamycin. Through these experiments we have revealed that variation in both the regiochemistry and relative stereochemical orientation of the substitution on a substituted cyclohexanecarboxylic acid are tolerated by the PKS in cases in which they are utilised as starter acids to initiate polyketide biosynthesis. A preference was observed for 1,4-hydroxyacids with a *trans* relationship. These results

Table 1. Incorporation of synthetic starter acids into rapamycin.

Starter acid analogue	Rapamycin analogue (Scheme 2)	Rapamycin analogue produced [mg L^{-1}]
		27 ± 2
		29 ± 2
 **11**		5 ± 1
 **12**		7 ± 1
 **13**		10 ± 1
 **16**		26 ± 2
 **18**		28 ± 1
 **21**		4 ± 1

suggest that the fluorinated cyclohexanecarboxylic acids previously prepared and fed to the same organism[8] did not result in fluorinated rapamycins because the strain was unable to hydroxylate the substrate due to the presence of a fluorine substituent.

Experimental Section

Full synthetic details and compound characterisation are described within the Supporting Information.

Feeding experiments: Compounds **11**–**13, 16, 18, 21**–**23** were prepared in methanol (0.32 mol L^{-1}) and were individually fed in quadruplicate to 24-hour-old *S. hygroscopicus* MG2–10 (BIOT-3016) cultures (50 μL to 7 mL culture, giving a final concentration of 2 mmol L^{-1}), except for compound **16**, which was fed in triplicate. The falcon tubes were shaken at 300 rpm (2.5 cm throw) at 26 °C for a further 5 days. A single sample of uninoculated media and two unfed *S. hygroscopicus* cultures were used as negative controls.

Analysis of fluorinated rapamycins: An aliquot of whole culture broth (0.9 mL) was added to methanol (0.9 mL) in a 2 mL Eppendorf, and then shaken for 30 min. The sample was centrifuged (10 min, 16 000 *g*) and the supernatant (150 μL) was transferred to an HPLC vial for analysis by HPLC with diode array detection. The HPLC system comprised an Agilent HP1100 equipped with a Hyperclone 3 μm BDS C18 130A column 150×4.6 mm (Phenomenex) heated to 50 °C. The gradient elution was from mobile phase B (55%) to mobile phase B (95%) over 10 min followed by an isocratic hold at mobile phase B (95%) for 2 min with a flow rate of 1 mL min^{-1}. Mobile phase A was 1:9 acetonitrile/water, containing ammonium acetate (10 mM) and trifluoroacetic acid (0.1%), mobile phase B was 9:1 acetonitrile/water, containing ammonium acetate (10 mM) and trifluoroacetic acid (0.1%). Rapamycin analogues were identified by the presence of the characteristic rapamycin triene, centred on $\lambda = 278$ nm. Samples were quantified based on a rapamycin calibration curve, measuring peak area at $\lambda = 280$ nm.

Preparation of 39-desmethoxy-39-fluoro-9-deoxo-16-*O*-desmethyl-27-desmethoxyrapamycin (1): Under sterile conditions MD6 media (2 L), D-fructose aqueous solution (40% (*w*/*v*), 100 mL) and filter sterilised L-lysine aqueous solution (14% (*w*/*v*), 28 mL) were mixed together to homogeneity. *S. hygroscopicus* seed cultures (0.5 mL) were used to inoculate portions (300×7.45 mL) of the MD6-fructose-L-lysine medium in 50 mL falcon tubes. The falcon tubes were sealed with foam bungs and were shaken at 300 rpm (2.5 cm throw) at 26 °C for 24 h.

Compound **18** was prepared in methanol to a concentration of 0.32 mol L^{-1} and was fed (50 μL, 16 μmol) to 300 previously prepared *S. hygroscopicus* MG2–10 (BIOT-3016) cultures to reach a final concentration of 2 mmol L^{-1}. The cultures were sealed with foam bungs and were shaken at 300 rpm (2.5 cm throw) at 26 °C for a further five days. The 300 cultures were combined, the cells harvested by centrifugation (25 min at 3480 *g*), and the supernatant was discarded. Acetonitrile (2.75 L) was added to the cells; they were stirred for one hour and decanted. The cells were extracted a second time with acetonitrile (2.4 L). The combined organics were reduced in vacuo to an aqueous slurry that was extracted with two volume equivalents of ethyl acetate. The solvent was then removed under reduced pressure to yield a crude extract (3.5 g).

The crude extract was dissolved in MeOH (50 mL) and silica (5 g) added. The solvent was removed in vacuo, and the resultant powder was loaded onto a column (40 g) of silica that was preconditioned with a mixture of ethyl acetate and hexanes (1:1). The column was then eluted with ethyl acetate/hexanes mixtures (1 L of 1:1; then 1 L 55:45; then 4 L of 60:40) and 250 mL fractions were collected. Fractions 5–20 were found to contain the target compound and were combined and the solvent removed in vacuo. The enriched extract was then dissolved in acetonitrile (1.8 mL) and purified by preparative HPLC (Column-Waters Xterra MS C18, 10 micron, 250 mm × 19 mm diameter; flow rate 20 mL min^{-1}; solvent A = water, solvent B = acetonitrile; isocratic 45% B, t_R of target compound = 21 min). The solvent was removed in vacuo to reveal the target compound as an off-white amorphous solid (16.4 mg).

CCDC 723198 (**18**-PNB) 723199 (**12**-PNB) and 723200 (**13**-PNB) contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif

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Keywords: antitumor agents · biosynthesis · fluorine · immunosuppressants · natural products

[1] a) L. M. Ballou, R. Z. Lin, *J. Chem. Biol.* **2008**, *1*, 27 – 36; b) R. T. Abraham, *Cell* **2002**, *111*, 9 – 12.
[2] D. E. Harrison, R. Strong, Z. D. Sharp, J. F. Nelson, C. M. Astle, K. Flurkey, N. L. Nadon, J. E. Wilkinson, K. Frenkel, C. S. Carter, M. Pahor, M. A. Javors, E. Fernandez, R. A. Miller, *Nature* **2009**, *460*, 392 – 395.
[3] I. R. Konings, J. Verweij, E. A. Wiemer, S. Sleijfer, *Curr. Cancer Drug Targets* **2009**, *9*, 439 – 450.
[4] For example see: a) K. C. Nicolaou, T. K. Chakraborty, A. D. Piscopio, N. Minowa, P. Bertinato, *J. Am. Chem. Soc.* **1993**, *115*, 9345; b) D. Romo, S. D. Meyer, D. D. Johnson, S. L. Schreiber, *J. Am. Chem. Soc.* **1993**, *115*, 7906; c) C. M. Hayward, D. Yohannes, S. J. Danishefsky, *J. Am. Chem. Soc.* **1993**, *115*, 9345; d) A. B. Smith, S. M. Condon, J. A. McCauley, J. L. Leazer, J. W. Leahy, R. E. Maleczka, *J. Am. Chem. Soc.* **1995**, *117*, 5407; e) M. L. Maddess, M. N. Tackett, H. Watanabe, P. E. Brennan, C. D. Spilling, J. S. Scott, D. P. Osborn, S. V. Ley, *Angew. Chem.* **2007**, *119*, 597 – 603; *Angew. Chem. Int. Ed.* **2007**, *46*, 591 – 597.
[5] a) A. Kirschning, F. Taft, T. Knobloch, *Org. Biomol. Chem.* **2007**, *5*, 3245 – 3259; b) S. Weist, R. D. Suessmuth, *Appl. Microbiol. Biotechnol.* **2005**, *68*, 141 – 150; c) R. Thiericke, J. Rohr, *Nat. Prod. Rep.* **1993**, *10*, 265 – 289.
[6] P. A. Lowden, G. A. Böhm, S. Metcalfe, J. Staunton, P. F. Leadlay, *ChemBioChem* **2004**, *5*, 535 – 538.
[7] M. A. Gregory, H. Petkovic, R. E. Lill, S. J. Moss, B. Wilkinson, S. Gaisser, P. F. Leadlay, R. M. Sheridan, *Angew. Chem.* **2005**, *117*, 4835 – 4838; *Angew. Chem. Int. Ed.* **2005**, *44*, 4757 – 4760.
[8] R. J. M. Goss, S. E. Lanceron, N. J. Wise, S. J. Moss, *Org. Biomol. Chem.* **2006**, *4*, 4071 – 4073.
[9] a) "Biomedical Frontiers of Fluorine Chemistry", K. L. Kirk, R. Filler, *ACS Symp. Ser.* **1996**, *639*, 1 – 24; b) S. Weist, B. Bister, O. Puk, D. Bischoff, S. Pelzer, G. J. Nicholson, W. Wohlleben, G. Jung, R. D. Süssmuth, *Angew. Chem.* **2002**, *114*, 3531 – 3534; *Angew. Chem. Int. Ed.* **2002**, *41*, 3383 – 3385; c) Z. Hojati, C. Milne, B. Harvey, L Gordon, M. Borg, F. Flett, B. Wilkinson, P. J. Sidebottom, B. A. M. Rudd, M. A. Hayes, C. P. Smith, J. Micklefield, *Chem. Biol.* **2002**, *9*, 1175 – 1188.
[10] K. Narasaka, H. Tanaka, F. Kana, *Bull. Chem. Soc. Jpn.* **1991**, *64*, 387 – 391.

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