One-step continuous flow synthesis of anti-fungal WHO Essential Medicine Flucytosine using fluorine

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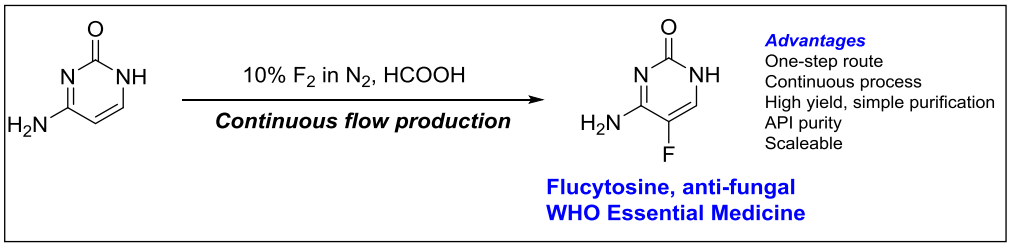


Abstract

In Africa around 625,000 mortalities per annum (20% of HIV/AIDS related deaths) are due to the affects of the *Cryptococcal meningitis (CM)* fungal infection. Recently, the World Health Organisation (WHO) and the Infectious Disease Society of America (IDSA) recommended that the first line treatment for *CM* is a combination of amphotericin B and Flucytosine, both now WHO Essential Medicines. However, Flucytosine is not even registered for use in any African nation due, in part, to its relatively high cost of manufacture and lack of generic manufacturers. Currently, Flucytosine is manufactured by an expensive four step manufacturing process. Here we report a one-step continuous flow process involving the reaction of inexpensive cytosine with fluorine gas using stainless steel tubular laboratory and pilot-scale silicon carbide reactor devices which is readily scaleable to a manufacturing process with low initial capital expenditure.

Keywords: *Cryptococcal meningitis*, fungal infection, flucytosine, essential medicine, fluorine, continuous flow

HIV/AIDS remains a major health issue in Africa where it is estimated that 2 million people die per annum from HIV or related diseases. Indeed, 625,000 mortalities[1,2] are due to the affects of *Cryptococcal meningitis (CM)*, a fungal infection that is of particular concern to HIV patients with heavily compromised immune systems and the leading cause of meningitis in Saharan Africa, responsible for 20% of HIV/AIDS related deaths world-wide.[3] Recently, the World Health Organisation (WHO) recommended that the first line treatment for *CM* is a combination of amphotericin B and Flucytosine **1** that results in a 39% decrease in 10-week mortality rate[4] and enhanced long term survival rates.[5] This led to a successful campaign from various health organisations to include Flucytosine in the most recent 19th WHO Core List of Essential Medicines (2015),[6] which lists medicines that must be available meet the minimum medical needs of a national health care programme. Flucytosine was approved by the FDA in 1971 (Ancoban®, Valeant) for the treatment of fungal infections and the only generic version was introduced to the US market in 2011 (Sigmapharm). In developed nations, 9% of patients diagnosed with CM die but this mortality rate increases dramatically to 70% of CM patients in sub-Saharan Africa where diagnosis and availability of suitable therapy is far too limited. Unfortunately, Flucytosine is not even registered for use in any African nation and, therefore, not available to prescribe for treatment.[3] Consequently, WHO, Medicines sans Frontieres and various national health organisations supported by reports from the Eurpean Molecular Biology Organisation (EMBO) workshop[7] have highlighted the urgent requirement for the supply of Flucyctosine to African nations. An 10-measure action plan has been suggested[3,7] including better global assessment, dissemination of best practice and diagnosis of the *CM* problem, increased registration, preferential pricing, licensing and supply of Flucytosine at affordable levels, to raise awareness and address this health issue. The recommended daily dosage of Flucytosine for a *CM* patient is 50-150 mg/kg and so, for a two week programme, each patient (body mass 50 kg) requires around 70 g of Flucyctosine to complete the course. It has been estimated that a 2-week inductive treatment of *CM* costs $62 for Flucytosine alone at the very least,[8] a prohibitive expense for low GDP nations.

In addition, Flucytosine **1** is an important intermediate required for the synthesis of Capecitabine **2** (anti-cancer) and Emtricitabine **3** (anti-HIV). Capecitabine (Xeloda®, Hoffman la Roche) was the first FDA approved oral chemotherapy drug (1998) with annual sales of

over $640 m and the first generic product was introduced in the US recently (September 2013, TEVA). Emtricitabine is also used in various formulations and combinational therapies for the treatment of HIV, the two best selling products being Atripla® (BMS, $2.9 bn sales in 2012) and Truvada® (Emtricitabine and Tenofovir; Gilead, $2.3 bn sales in 2012).

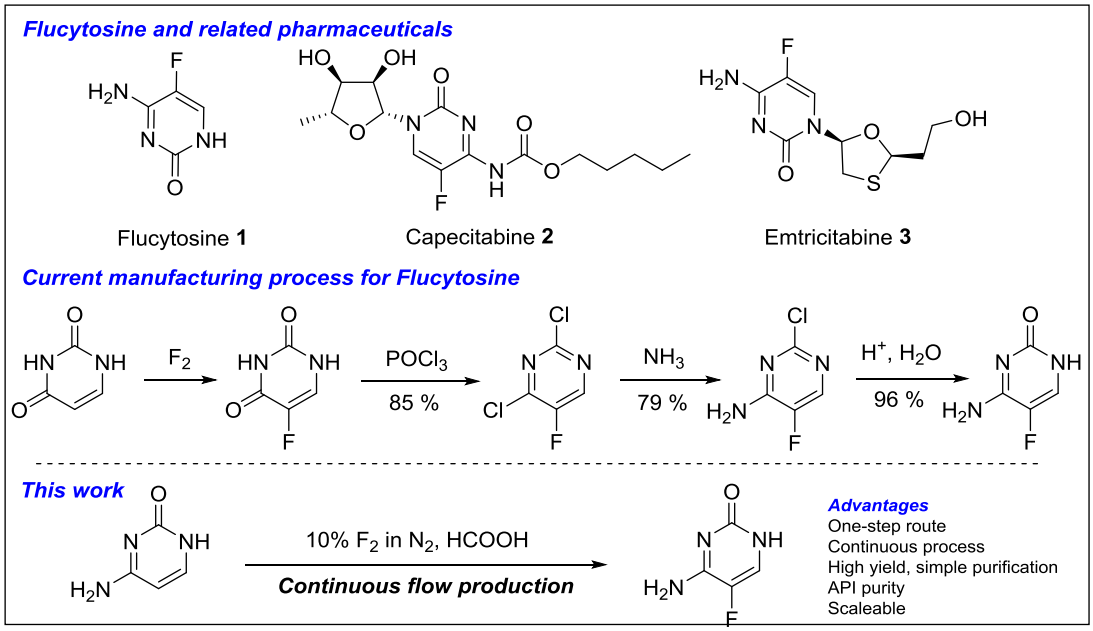


Figure 1: *Flucytosine **1**: related pharmaceuticals and synthesis*

Increased availability of Flucytosine to patients in less developed nations requires a much reduced cost of manufacture of Flucytosine. Market failure in Africa has, in part, been attributed to the relatively high cost of Flucytosine because of a lack of generic competition in low income markets[9] possibly due to low profit margins associated with current costs of Flucytosine manufacture. Reported currently used 4-step manufacturing routes[10] to Flucytosine that are described in the patent literature utilise 5-fluorouracil as the starting material and require chlorination, amination and hydrolysis stages to complete the 4 stage synthesis of Flucytosine from uracil.[11,12] Fluorine gas is used for the large scale manufacture of a fluoropyrimidine derivative as an intermediate in the synthesis of Pfizer's V-Fend (Voriconazole) antifungal agent[13] as well for the production of 5-fluorouracil, a long established effective anti-cancer drug for solid tumour treatment.[14] Consequently, fluorine gas is used on the manufacturing scale when appropriate reaction selectivity and cost benefits for a given process can be achieved.

A simplified method suitable for scale-up to manufacturing quantities of Flucyctosine could, in principle, be the direct fluorination of inexpensive cytosine using cheap fluorine gas. However, a suitable, scaleable direct fluorination synthesis of Flucytosine has not been reported in the literature.[15,16] In this paper, we describe an inexpensive and readily scaleable method for the synthesis of Flucytosine from cytosine using fluorine gas as part of a long term programme developing the use of fluorine gas as a reagent for organic chemistry using both batch and continuous flow reaction techniques.[17-20]

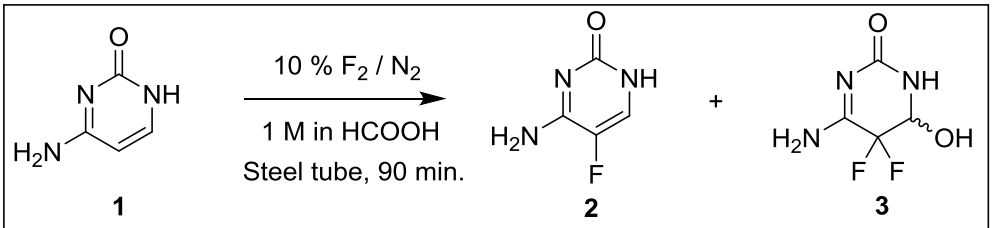
Initially, we examined fluorination of cytosine by fluorine using a conventional batch process. Cytosine is very soluble in formic acid (160 g/L [1.45 M]), a preferred medium for direct fluorination due to enhanced reaction control for electrophilic fluorination processes.[21] Batch fluorinations were conducted by passing fluorine gas (10% in nitrogen) via a gas flow meter through a solution of cytosine in formic acid at room temperature. Following work-up, a crude mixture that contained mainly flucytosine (*approx.* 50 % by ^{19}F NMR spectroscopy, δ -170 ppm, doublet) and one major difluorinated side product (δ – 114 (dd, $^{2}J_{FF}$ 279 Hz, $^{3}J_{HF}$ 6 Hz) – 131 ppm (dd, $^{2}J_{FF}$ 279 Hz, $^{3}J_{HF}$ 3 Hz)) along with several other side products was obtained, consistent with earlier reports.[16] Recrystallization of the crude product mixture from water efficiently removed all difluorinated organic impurities, but the separation of unreacted starting material from the desired flucytosine product proved to be very difficult. Therefore, the reaction conditions were adapted to give 100 % conversion by passage of an excess of fluorine gas through the rapidly stirred reaction mixture (Table 1). Consequently, after addition of 1.75 equivalents of fluorine, work-up and simple recrystallization from water, pure Flucytosine was isolated in reasonable yield (38 %).

Our preliminary results, therefore, demonstrated that batch fluorination processes can be used to synthesise Flucytosine in moderate yield although the formation of significant quantities of difluorinated product leads to considerable waste which would contribute to higher production costs. As an alternative to batch manufacturing processes, there are many advantages associated with using continuous flow reactors for chemical synthesis and among those discussed widely[22-24] including high throughput, reduced waste streams, low manufacturing, operation and maintenance costs, low power consumption, increased precision and process control. Flow reactors may also lead to increased reaction selectivity due to optimisation of contact between reagents because of very rapid mixing in such

devices and laboratory and manufacturing scale continuous flow systems are now commercially available.[24] Consequently, we studied the fluorination of cytosine using a continuous flow reactor system where greater fluorination reaction control may be possible.

While we have utilised various laboratory continuous flow reactors previously for direct fluorination processes,[19] for this application we used a narrow-bore stainless steel tube (1.4 mm ID, 1 m length) as the continuous flow reaction vessel that ensures good gas-liquid mixing and is operationally simple. Consequently, fluorine gas (10% in nitrogen) was passed through the steel tube via gas flow meter at the same time as a solution of cytosine in formic acid was introduced into the tube reactor at a prescribed rate by syringe pump (Table). After some optimisation, involving changing flow rates and equivalents of fluorine gas added, 100% conversion of the starting material was observed and Flucytosine was isolated (63%) by recrystallization indicating the feasibility of using flow reactors for Flucytosine manufacture.

Table 1: *Synthesis of Flucytosine*



Cytosine **1**	Fluorine	Conversion	**2** : **3**	Yield **2**
Batch conditions				
1.0 equiv.	1.75 equiv.	>99%	44 : 56	38%
Steel tube flow reactor				
4 mmol h^{-1}	4 mmol h^{-1}	95%	93 : 7	66%
4 mmol h^{-1}	4.5 mmol h^{-1}	98%	93 : 7	64%
4 mmol h^{-1}	5 mmol h^{-1}	>99%	95 : 5	63%
Pilot Scale Silicon Carbide continuous flow reactor				
0.54 mol h^{-1}	0.7 mol h^{-1}	>99%	95 : 5	83%*

* Isolated 59 g of Flucytosine (>99.9% purity) after one hour flow reaction
See Supporting Information for details of laboratory equipment and experimental procedures

Scale-up of our initial laboratory scale experiments was conducted using a *meso*-scale Boostec® flow reactor system[25] fabricated from silicon carbide, a material that is both resistant to the corrosive reaction medium (fluorine, HF and acidic solvent) and has excellent heat exchange properties appropriate for controlling exothermic processes. For the fluorination of cytosine, a reactor built from six process plates was used giving a 16 m total channel length and 61 mL reactor volume. After process development studies, cytosine solution in formic acid (10 wt%, 600 g/h) and fluorine (10 vol. % in nitrogen, 204 g/h) were passed through the continuous flow silicon carbide reactor via flow meters (Figure 2) which was externally cooled to 10 ^{o}C and, after reaching steady state (15 minutes), the product liquid phase was collected over a 60 minute time period to yield a yellow solution (620 g). *n*-Butanol was added to the product mixture to precipitate the product and, after filtration, flucytosine was isolated (58.0 g, 99.8 % purity by HPLC, 83 % yield).

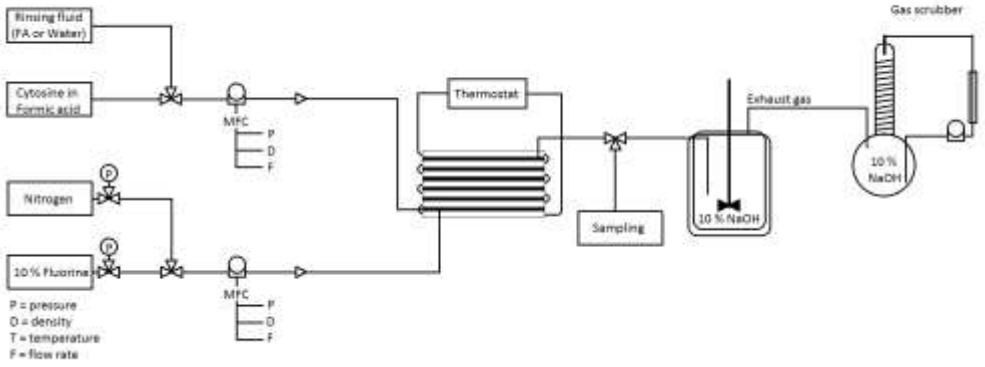


Figure 2: Continuous flow reactor system for manufacture of Flucytosine

In summary, a one-step synthesis of Flucytosine from cytosine using continuous flow direct fluorination techniques has been established and transferred to the pilot scale (60 g production of pure Flucytosine per hour per single reaction channel). This operationally simple procedure from inexpensive starting materials offers the only alternative manufacturing procedure for Flucytosine to the currently operated expensive four step process since flow reactors fabricated from silicon carbide are available that enable larger scale production.[25] We envisage that this one-step low cost synthesis of Flucytosine will help to raise awareness of the neglected *CM* health epidemic and ultimately contribute to meeting the urgent requirement for large quantities of Flucytosine for low income nations as stated by WHO, IDSA and Medicins sans Frontieres.[3,7]

Methods

Pilot scale continuous flow fluorination synthesis of Flucytosine 1

Cytosine solution in formic acid (10 wt%, 600 g/h) and fluorine (10 vol. % in nitrogen, 204 g/h) were passed through the silicon carbide Boostec® flow reactor system[25] at 10 ^{o}C and after reaching steady state (15 minutes) the product liquid phase was collected for 60 minutes to yield a yellow solution (620 g). To this solution *n*-butanol (1000 g, 1.23 L) was added in five portions (no exotherm observed) and the mixture was stirred at rt for 12 h. After filtration, the solid was washed once with *n*-butanol (60 mL) and dried in air to leave

Flucytosine (58.0 g, 99.8 % by HPLC, 83 % yield); M.p.: 295 - 300 °C (decomposes), ($[M]^+$ 129.0337, $[M]^+$ requires: 129.0338); IR (cm^{-1}): 3384, 3092, 2724, 1665, 1624, 1551, 1454, 1216; ^1H NMR (400 MHz, D_2O+DCl) 7.83 (1H, d, $^3J_{HF}$ 4.8 Hz); ^{19}F NMR (400 MHz, D_2O+DCl) -169.7 (1F, d, $^3J_{HF}$ 4.8 Hz); ^{13}C NMR (100 MHz, D_2O+DCl): 130.67 (d, $^2J_{CF}$ 29.6 Hz), 135.25 (d, $^1J_{CF}$ 232 Hz), 147.88, 153.65 (d, $^2J_{CF}$ 23.4 Hz); MS (ASAP): 111 (37 %, $[M+H-F]^+$), 129 (8 %, $[M]^+$), 130 (100 %, $[M+H]^+$); physical and spectral data consistent with an authentic sample of Flucytosine.

Supporting Information.

Details of selective direct fluorination processes using conventional batch, stainless steel tube and silicon carbide continuous Flow Reactor protocols and spectroscopic data accompanies this paper.

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Author Contributions

All authors conceived and designed the experiments: A. H. and A.C. performed the experiments: A.H, A.C, and S.G. analyzed the data: A.H and G.S. co-wrote the paper.

Competing Interests

The authors declare that they have no competing financial interests.

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References

1. Armstrong-Jones, D., Meintjes, G. & Brown, G.D. A neglected epidemic: fungal infections in HIV/AIDS. *Trends in Microbiology* **22** 120-127 (2014).

2. Park, B.J., Wannemueller, K.A., Marston, B.J., Govender, N., Pappas, PG. & Chiller, T.M. Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. *AIDS* **23**, 525-530 (2009).

3. Loyse, A., Thangaraj, H., Easterbrook, P., Ford, N., Roy, M., Chiller, T., Govender, N., Harrison, T. S. & Bicanic, T. Cryptococcal meningitis: improving access to essential antifungal medicines in resource poor countries. *Lancet Infect. Dis.* **13**, 629–637 (2013).

4. Day, J.N., Chau, T.T.H., Wolbers, M., Mai, P.P., Dung, N.T., Mai, N.H., Phu, N.H., Nghia, H.D., Phong, N.D., Thai, C.Q., Thai, L.H., Chuong, L.V., Sinh, D.X., Duong, V.A., Hoang, T.N., Diep, P.T., Campbell, J.I., Sieu, T.P.M., Baker, S.G., Chau, N.V.V., Hien, T.T., Lalloo, D.G. & Farrar, J.J. Combination antifungal therapy for cryptococcal meningitis. *N. Engl. J. Med.* **368**, 1291-1302 (2013).

5. Butler, E.K., Boulware, D.R., Bohjanen, P.R. & Meya, D.B. Long term 5-year survival of persons with cryptococcal meningitis or asymptomatic subclinical antigenenia in Uganda. *PLoS ONE*, **7**, e51291 (2012).

6. WHO. WHO Model List of Essential Medicines, 19th Edition (April 2015). Available at: http://www.who.int/medicines/publications/essentialmedicines/EML2015_8-May-15.pdf. Accessed May 20th 2016.

7. Brown, G.D., Meintjes, G., Kolls, J.K., Gray, C. & Horsnell, W. AIDS-related mycoses: the way forward. *Trends in Microbiology* **22**, 107-109 (2014).

8. Bartlett, J.G., Auwaerter, P.G. & Pham, P.A. Johns Hopkins ABX Guide: Diagnosis and Treatment of Infectious Disease, 3rd Edition, 2012.

9. Loyse, A., Dromer, F., Day, J., Lortholary, O. & Harrison, T.S. Flucytsoine and cryptococcosis: time to urgently address the worldwide accessibility of a 50-year-old antifungal. *J. Antimicrob. Chemother.* **68**, 2435-2444 (2013).

10. Kleeman, A., Engel, J., Kutscher, B. & Reichert, D. *Pharmaceutical Substances: Syntheses, Patents, Applications of the most relevant APIs*, 5th ed., Thieme, Stuttgart (2008).

11. Duschinsky, R. Process and intermediates for deoxyfluorocytidine. US Pat. 3040026 (1962).

12. Duschinsky, R. 2-Chloro-4-amino-5-fluoropyrimidine. US Pat. 3185690 (1965).

13. Butters, M., Ebbs, J., Green, S. P., MacRare, J., Morland, M. C., Murtiashaw, C. W. & Pettman, A. J. Process Development of Voriconazole: A Novel Broad-Spectrum Triazole Antifungal *Agent. Org. Proc. Res. Dev.* **5**, 28-36 (2001).

14. Schuman, P. D., Tarrant, P., Warner, D. A. & Westmoreland, G. Process for fluorinating uracil and derivatives thereof. US 3954758 (1976).

15. Takahara, T. Process for preparing 5-Fluorocytosine salt. US Pat. 4473691 (1984).

16. Visser, G. W., Boele, S., Van Halteren, B. W., Knops, G. H., Herscheid, J. D., Brinkman, G. A. & Hoekstra, A. Mechanism and Stereochemistry of the Fluorination of Uracil and Cytosine Using Fluorine and Acetyl Hypofluorite. *J. Org. Chem.* **51**, 1466–1471 (1986).

17. Chambers, R.D., Fox, M.A., Holling, D., Nakano, T., Okazoe, T. & Sandford, G. Elemental Fluorine. Part 16. Versatile thin-film gas-liquid multi-channel microreactors for effective scale-out. *Lab. Chip* **5**, 191-198 (2005).

18. Chambers, R.D., Fox, M.A. & Sandford, G. Elemental Fluorine. Part 18. Selective direct fluorination of 1,3-Ketoesters and 1,3-diketones using microreactor technology. *Lab. Chip* **5**, 1132-1139 (2005).

19. McPake, C.B. & Sandford, G. Selective continuous flow processes using fluorine gas. *Org. Proc. Res. Dev.* **16**, 844-851 (2012).

20. Harsanyi, A. & Sandford, G. Fluorine gas for Life Science syntheses: Green metrics to assess selective direct fluorination for the synthesis of 2-fluoromalonate esters. *Green Chem.* **17**, 3000-3009 (2015).

21. Chambers, R.D., Skinner, C.J., Hutchinson, J. & Thomson, J. Elemental fluorine. Part 1. Synthesis of fluoroaromatic compounds. *J. Chem. Soc., Perkin Trans. 1* 605-609 (1996).

22. Wiles, C. & Watts, P. *Micro Reaction Technology in Organic Synthesis*, CRC-Press (2011).

23. Hartwig, J., Metternich, J. B., Nikbin, N., Kirschning, A. & Ley, S.V. Continuous flow chemistry: a discovery tool for new chemical reactivity patterns. *Org. Biomol. Chem.* **12**, 3611−3615 (2014).

24. Fitzpatrick, D.E., Battilocchio, C. & Ley, S.V. Enabling Technologies for the Future of Chemical Synthesis. *ACS Cent. Sci.* **2**, 131–138 (2016).

25. Continuous flow reactors from the Corning company. Available at: http://www.corning.com/media/worldwide/global/documents/G1_SiC_leaflet_FINAL_6.1.15.pdf; http://www.corning.com/media/worldwide/global/documents/G4Reactor_6.9.15.pdf