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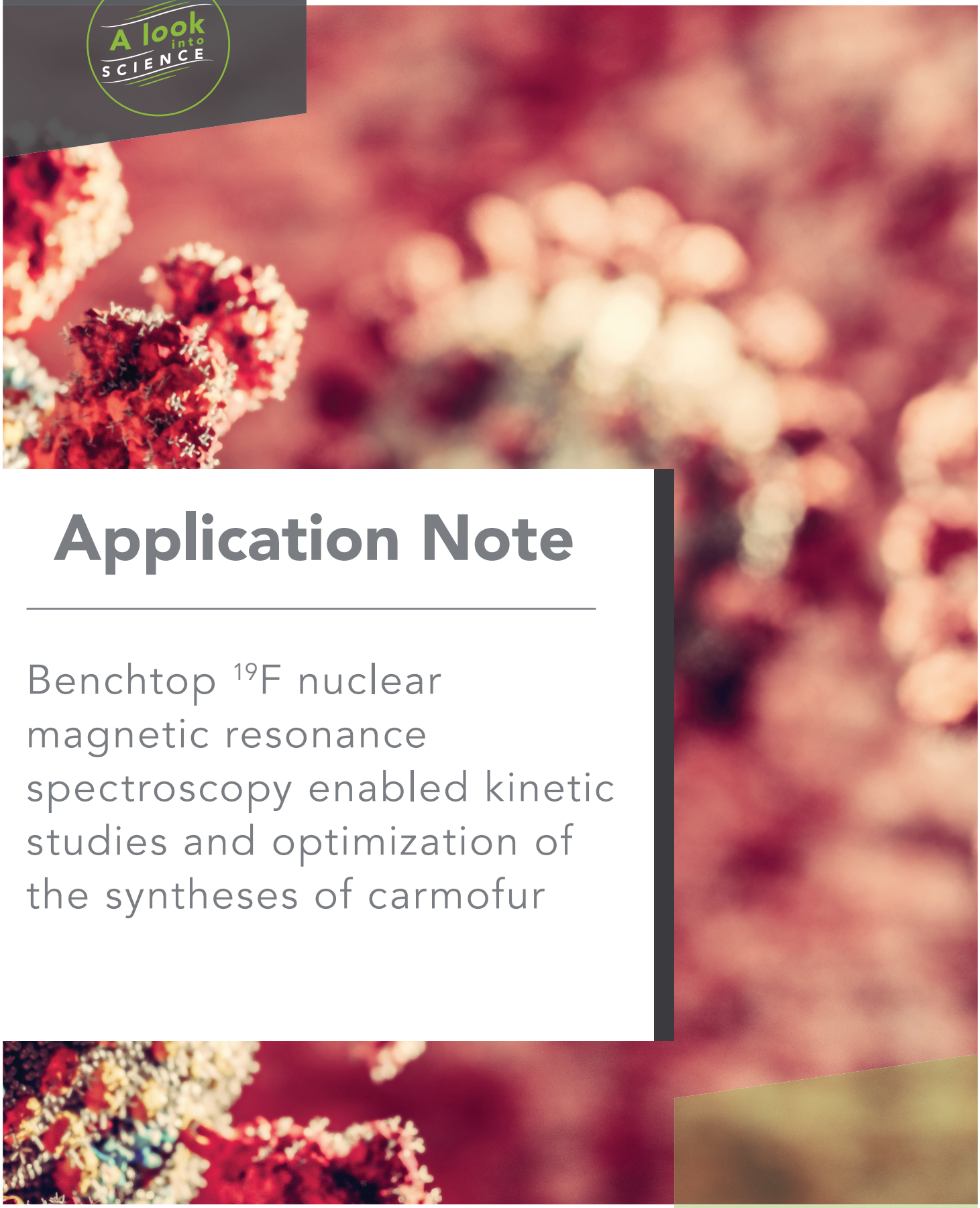

Application Note

Benchtop ^{19}F nuclear magnetic resonance spectroscopy enabled kinetic studies and optimization of the syntheses of carmofur

APPLICATION NOTE
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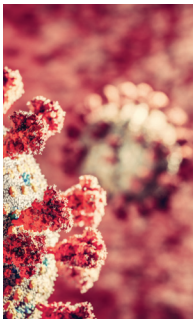

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Introduction

Carmofur, a small molecule derivative of 5-fluorouracil (5-FU), has been used as an antineoplastic agent against colorectal cancer for several decades and has been recently identified through drug repurposing efforts as a potent covalent inhibitor of SARS-CoV-2 main protease (M^{pro}) activity, making it a novel and promising lead compound for the development of antivirals to combat COVID-19.[1-3]

While carmofur can be synthesized by treating 5-FU with phosgene and hexylamine, more efficient yields were observed by coupling 5-substituted uracils with *n*-hexyl isocyanate conducted in pyridine (**Figure 1**). However, previously recorded synthetic procedures have suffered from low yields and inefficient reaction times.[4-7] Additionally, due to the limited solubility of 5-fluorouracil in most organic solvents, this reaction must be performed in exceptionally polar, non-volatile solvents such as pyridine, making it inconvenient to monitor by thin-layer chromatography. Fortunately, carmofur's fluorine atom serves as a spectroscopic handle for benchtop ^{19}F nuclear magnetic resonance spectroscopy (NMR) to kinetically monitor the reaction of 5-FU with various isocyanates.

Recent advances in benchtop NMR instrutemnts have facilitated real-time reaction monitoring, characterization, and quantification of chemical entities, becoming a practical analytical tool for precise structural elucidation, reaction condition optimization, tracking impurities or yield, as well as reaction kinetics directly in the laboratory.[8,9] Furthermore, proton lock capabilities on benchtop NMR spectrometers have obviated the need for deuterated solvents, which enabled us to monitor reactions in non-deuterated pyridine. Additionally, spectra can be acquired in mere seconds directly in a synthetic laboratory, and therefore this workflow can be used for rapid and real-time quantification of chemical entities in a crude reaction mixture without modification of its reaction conditions. Most importantly, it can provide quantitative insight to rapidly screen and optimize reaction conditions.

Here, we implement benchtop ^{19}F NMR spectroscopy to quantitatively track and optimize the synthesis of carmofur. We show that this reaction can be performed on a benchtop NMR spectrometer directly in a synthetic chemistry laboratory, and that this workflow can be used for real-time quantitative reaction monitoring in the synthesis of carmofur in a manner that is less invasive and time consuming than other conventional methods for analytical quantitation such as high-performance liquid chromatography (HPLC) or mass spectrometry. This solution has enabled the scale of such reactions to produce gram quantities of these compounds, as well as the convenient and efficient collection of ^{19}F spectroscopic data.

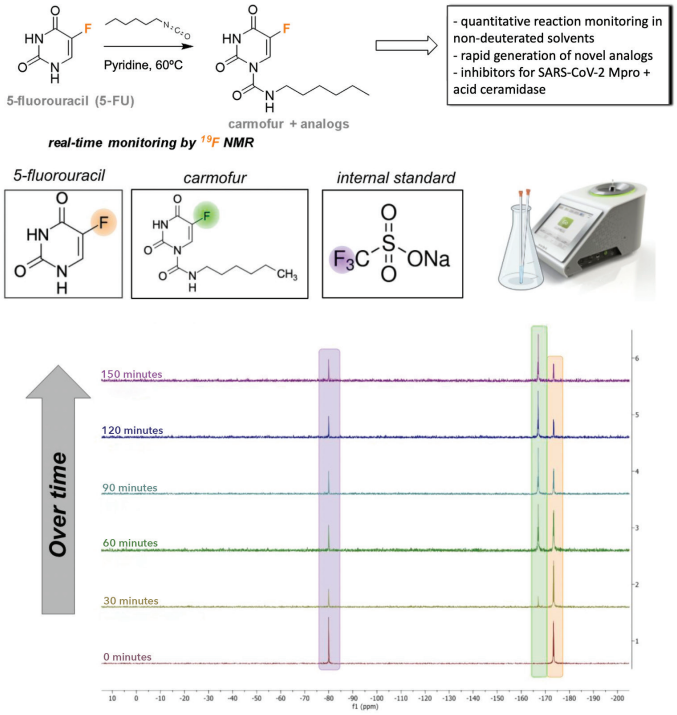

***Figure 1.** A time course is generated through kinetic monitoring by ^{19}F NMR spectroscopy, where real-time formation of carmofur can be observed.*

Materials and Methods

Synthetic Methods

A 0.1 M stock solution of 5-fluorouracil (1 eq, 1 mmol) with the internal standard was prepared with anhydrous solvent and added to an NMR tube. Hexyl isocyanate (1.5 eq, 0.105 mmol) was subsequently added, and the NMR tube headspace was flushed with nitrogen. The reaction mixture was placed into a 60 °C water bath and was periodically monitored at 30-minute intervals for 3 hours. All NMR tubes were sealed using teflon tape and parafilm due to carmofur's sensitivity to moisture, and each reaction was conducted in triplicate to ensure reproducibility.

Physical Methods

^{19}F NMR spectra of compounds were collected on a Nanalysis 60PRO benchtop NMR spectrometer. Sodium trifluoromethanesulfonate (0.2 eq, 0.014 mmol) was used as the internal calibrant because it is thermally stable to reaction conditions and is well-separate from the analyte peaks. The concentrations of reactant and product were quantified by ^{19}F NMR by integrating the sodium trifluoromethanesulfonate reference peak at -79.1 ppm and the ^{19}F resonance of 5-fluorouracil (-172 ppm) and product (-166 ppm) over time. Final ^{1}H NMR spectra of synthesized carmofur product were collected in chloroform-*d*. NMR spectra were processed and visualized on the MestreNova software package (version 14.2.3).

Results and Discussion

Effects of Base

Since previous studies have concluded that isocyanate reactions can be accelerated by basic catalysis and that treating them with base can greatly increase its rate of nucleophilic addition, common organic bases such as 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), 1,4-diazabicyclo[2.2.2]octane (DABCO), 4-dimethylaminopyridine (DMAP), and triethylamine (TEA) were added to the reaction mixture.[10,11] However, the addition of DBU or DMAP led to rapid decomposition of carmofur produced in the reaction and the addition of TEA or DABCO did not lead to productive conversion.

Effects of Temperature

In efforts to optimize the conditions to synthesize carmofur, various temperatures were evaluated to determine the reaction rate in pyridine. The reaction progressed to nearly 72% NMR yield at 45 °C, nearly double the yield of previously reported synthetic conditions.[4-7] Although high temperatures had rapid initial conversion, it led to product decomposition over time (**Figure 2**).

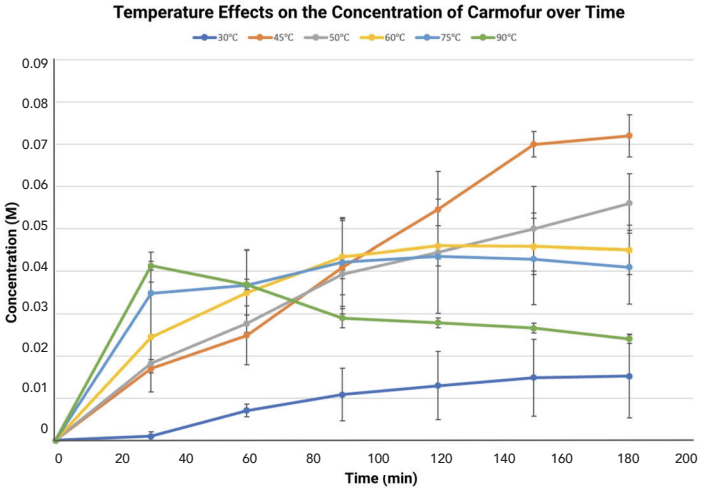

Figure 2. *Concentration of carmofur over time with various temperatures in pyridine. 45 °C was found to be the optimal temperature.*

Effects of Moisture

It was found that this reaction is extremely sensitive to water since isocyanates react with water to form carbon dioxide, polyurethanes, and ureas.[12] In general, the higher amounts of trace water in the reaction, the slower the overall reaction rate, showing the necessity of conducting this reaction with rigorous sealing techniques to ensure it is moisture-free (**Figure 3**).

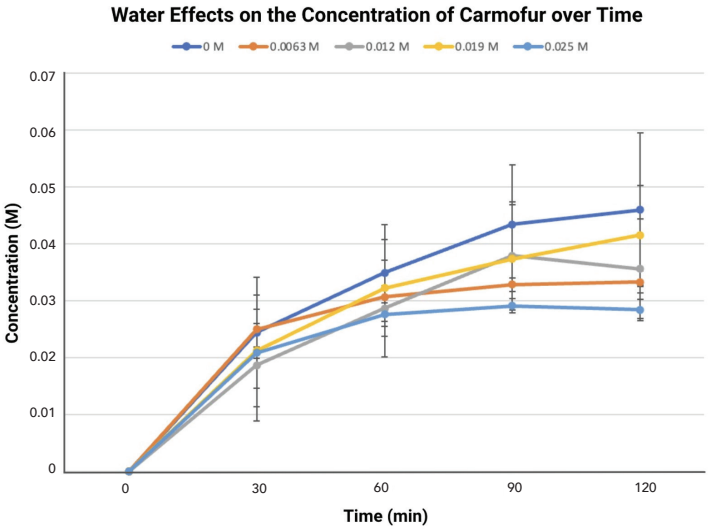

Figure 3. *Concentration of carmofur over time with increased concentrations of water in the reaction.*

Solvent Screening

Historically, the solvents used to synthesize carmofur include pyridine, dimethylformamide (DMF), and dimethyl sulfoxide (DMSO).[4-7] To test how solvation affected the reaction, various polar, aprotic solvents were evaluated. At 60 °C, the reactions performed in *n*-methyl-2-pyrrolidone (NMP) and pyridine had comparable endpoint yields (**Figure 4**); however, pyridine is carcinogenic and has high ecotoxicity, while NMP is non-carcinogenic and has low acute toxicity via oral, dermal, and inhalation exposures.[13,14] Additionally, NMP is readily removed through aqueous extraction when synthesizing carmofur on-scale, making NMP the ideal solvent of the two.

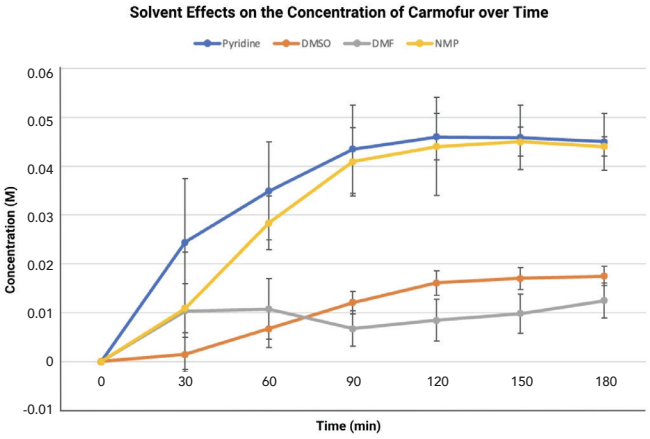

Figure 4. *Concentration of carmofur over time with various solvents. NMP was found to have comparable yields as pyridine, which has enabled more convenient scale-up of carmofur and its related analogs.*

Conclusion

We showed the applicability of reaction monitoring by ^{19}F NMR for reaction optimization by determining the optimal conditions for synthesizing carmofur in temperatures ranging from 40-50 °C in pyridine. More importantly, through a screen of dozens of reaction conditions in non-deuterated solvent, we were able to identify *n*-methyl-2-pyrrolidinone (NMP) as a solvent that is easier, and safer to synthesize carmofur on-scale with comparable yields to previously reported conditions. Altogether, these improvements to the synthesis of carmofur have allowed for a more efficient and safer synthesis of carmofur on scale and demonstrate that it is possible to quantitatively track a reaction by benchtop ^{19}F NMR of crude reaction mixtures in non-deuterated solvents. We envision this workflow can be applied in future studies as a convenient method to efficiently track the synthesis of related 5-FU analogs and other fluorinated bioactive substrates.

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