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Employment of on-line FT-IR spectroscopy to monitor the deprotection of a 9-fluorenylmethyl protected carboxylic acid peptide conjugate of doxorubicin

Mark Cameron *, George X. Zhou [1], Micheal B. Hicks, Vincent Antonucci, Zhihong Ge, David R. Lieberman, Joesph E. Lynch, Yao-Jun Shi

Department of Process Research, Merck Research Laboratories, Merck & Co. Inc., PO Box 2000, Rahway, NJ 07065, USA

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Abstract

A method for accurately determining the end-point, $>98\%$ conversion, of the deprotection reaction of a highly toxic 9-fluorenylmethyl (Fm) ester **1b** to its corresponding carboxylate **1d** in real time by FT-IR spectroscopy is reported. Advantages of this method over analysis by conventional chromatographic means include real time determination of the end-point of a reaction that is time sensitive to by-product formation, and elimination of sampling a highly toxic reaction mixture. The FT-IR method is based on monitoring, in real time, the disappearance of the Fm ester carbonyl band for **1b** at 1737 cm^{-1}, during deprotection by piperidine, and calibration models were established by Partial Least Squares (PLS) regression analysis with high performance liquid chromatography (HPLC) as reference. The best calibration model was built with 5 PLS factors in the spectral range of 1780–1730 and 1551–1441 cm^{-1} and resulted in a standard error of cross validation (SECV) of 0.63 mM **1b** and a standard error of prediction (SEP) of 0.51 mM **1b** in the range of 0–25 mM. This error of prediction is approximately 0.8% of the initial concentration of **1b** and is well within our specifications of $<2\%$ initial concentration.

Keywords: On-line; FT-IR spectroscopy; Doxorubicin peptide conjugate; 9-fluorenylmethyl ester deprotection; End-point determination

1. Introduction

In the pharmaceutical industry synthetic processes require rapid and reliable analytical methods to determine the composition of reaction mixtures and reaction end-points. This is commonly achieved satisfactorily by chromatographic means such as high performance liquid chromatography (HPLC), gas chromatography (GC)

* Corresponding author. Tel.: +1-732-594-0927; fax: +1-732-594-8360.
E-mail address: mark_cameron@merck.com (M. Cameron).
[1] Also corresponding author.

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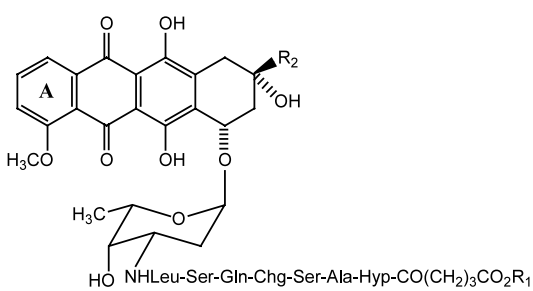
and thin liquid chromatography (TLC). However, these techniques often require time consuming handling of numerous samples, as well as, assays typically requiring tens of minutes. This becomes problematical if the reaction is time sensitive to by-product formation and if the reaction mixture comprises of a highly potent compound, when minimal handling is a premium. Consequently, the development of any method that permits the in situ real-time assay of these compounds undergoing a chemical transformation would be extremely advantageous. With the advances in technology FT-IR spectroscopy has gained increasing recognition [1–5] for rapid in situ analysis of chemical reactions and has proven to be a valuable technique in monitoring reaction intermediates [6–8], and products [9,10]. During our investigation into the large-scale synthesis of a cytotoxic prostate cancer drug candidate **1a**, we desired an on-line method of assay for the conversion ($>98\%$) of **1b**–**1d** (Scheme 1). This was because of the highly toxic nature of the reaction mixture, as well as, time dependent formation of difficult to remove, by non-chromatographic means, by-products, such as, **1e** [11]. Since FT-IR is capable of handling both corrosive and highly toxic mixtures, as well as, affording real time information about a reaction it appeared to be the ideal choice to replace our existing time consuming HPLC assay that required handling of numerous samples under stringent safety protocols. We now wish to report our preliminary results that show the applicability of on-line FT-IR spectroscopy for monitoring a key step in the synthesis of a pharmaceutically important prostate cancer drug candidate [12].



1a, $R_1 = H$; $R_2 = COCH_2OH$
1b, $R_1 = Fm$; $R_2 = COCH_2OH$
1c, $R_1 = Na^+$; $R_2 = COCH_2OH$
1d, $R_1 = C_5H_{10}NH_2^+$; $R_2 = COCH_2OH$
1e, $R_1 = Na^+$; $R_2 = COOH$

Scheme 1.

2. Experimental

2.1. Apparatus and materials

A FT-IR instrument (ReactIR 1000) equipped with an ATR probe from ASI Applied Systems, Millersville, MD was used. The software package REACTIR2 (version 2.22) was used to control the instrument and collect spectra. Each spectrum was collected as absorption spectrum of 32 co-added scans with nitrogen as background in the spectral range 4000–650 cm^{-1} in every minute over the course of reaction. Happ–Genzel apodization and a spectral resolution of 4 cm^{-1} were adopted. The resulting reaction spectra were deconvoluted with CONCIRT (version 2), an add-on software package of this instrument by ASI Applied Systems for component spectra and their corresponding profiles. Multivariate regression analysis was performed with the software package GRAMS/32 enhanced with an add-on subroutine PLSPLUS/IQ[2] (Galactic Industries Corporation, Salem, NH). Liquid chromatographic analyses were performed on a HP 1100 Series (Hewlett Packard, Wilmington, DE). Anhydrous HPLC grade DMF and reagent grade piperidine were obtained from Aldrich, St. Louis, MO).

2.2. Deprotection

The reaction was studied in the laboratory employing glassware modified to accept the ATR probe. To a mechanically stirred, cooled (-5 °C) solution of **1b** in DMF (61.5 mM), was inserted the ATR probe, and spectra acquisition initiated at minute intervals. After a few minutes piperidine (1.5 eq) was added and the reaction was stirred at -5 °C over 3 h. Samples of the reaction mixture were taken during the course of the reaction for HPLC assay and correlated against the appropriate spectral acquisition.

2.3. HPLC analysis

The HPLC assay for determination of **1b** concentration in the reaction mixture was performed on a Zorbax XBD-C18 (250×4.6 mm, 5μ) column at 25 °C, and components detected at 234 nm. Separation was achieved employing a gradient elution of two mobile phases at 0.6 ml min^{-1}. Phase A consisted of 0.1% w/v ammonium acetate (adjusted to pH 4.4 by the addition of acetic acid) and phase B acetonitrile. The amount of B delivered increased form 30 to 55% over 40 min. Under these conditions **1a** eluted after 12.9 min and **1b** after 36.0 min.

2.4. Data analysis

Calibration models were built with partial least squares (PLS) regression on PLSPLUS/IQ, an add-on program of GRAMS/32 (Galactic Industries Corporation). During the calibration step, a full cross validation (leave-one-out) was applied with calibration data set and the standard error of cross validation (SECV) was obtained for each calibration model [13]. The model with the lowest SECV was taken to be the optimum and used to predict the separate prediction data set. The model that provided the lowest standard error of prediction (SEP) on the prediction set was chosen as optimum [13].

$$\mathrm{SECV} = \sqrt{\frac{\sum_{i=1}^{m} (\hat{c}_i - c_i)^2}{m}} \qquad \mathrm{SEP} = \sqrt{\frac{\sum_{i=1}^{m} (\hat{c}_i - c_i)^2}{m-1}}$$

Where, c_i and $\hat{c}_i$ are the actual, and estimated or predicted concentrations, respectively, while m is the number of samples in the calibration or prediction set.

3. Results and discussion

3.1. Spectral features

The FT-IR spectra of both **1b** and the carboxylate **1c** in the reaction solvent DMF were compared in order to determine, after subtraction of DMF, spectral differences between either **1b** and its corresponding carboxylate that could be used to monitor the reaction. The sodium salt **1c** was used for this purpose since the piperidine salt **1d** is not stable to isolation. It was observed (Fig. 1) that in the region of aromatic C–H out of plane bending region **1b** exhibited two strong absorption bands at 768 and 745 cm^{-1}. These signals were attributed to an out of plane bend for the aromatic C–H bonds of the fluoroenyl methyl group, with some contribution to the intensity at 768 cm^{-1} from the A ring of the Doxorubicin moiety of the molecule. In contrast **1c** exhibited a weak aromatic C–H out of plane bend absorption from the A ring of the Doxorubicin moiety at 768 cm^{-1}. Upon addition of piperidine to a solution of **1b** in DMF the intensity of signals at 768 and 745 cm^{-1} began to decrease and a new set at 788 and 740 cm^{-1} was generated. This new set was attributed to an out of plane bend for aromatic C–H bonds of the dibenzofulvene **2** (Scheme 2). However, it was interesting to note that over the course of the reaction the signals at 768 and 745 cm^{-1} did not completely disappear even when a three fold excess of piperidine was used but rather reached a minimum (Fig. 2) and then began to increase in intensity. Conversely, the signal intensities at 788 and 740 cm^{-1} reached a maximum and then decreased. Our previous examination of this reaction by HPLC had shown that the reaction did not go to completion with only a theoretical stoichiometric, i.e. one mol equivalent, amount of piperidine, but required at least two mol equivalents in order to achieve complete reaction i.e. $< 1\%$ of starting material remaining. A possible explanation for this result may be the consumption [14,15] of piperidine by **2** to give the hindered tertiary amine **3** (Scheme 2), hindered amines were found to be particularly poor in causing deprotection of **1b,** for which the out of plane bend for the aromatic C–H bonds are unresolved from those of **1b**. Obviously, the end of the reaction could not be determined by monitoring the decrease or increase in intensity of either set of signals for out of plane bend of the aromatic C–H bonds of **1b** or **2**. Upon further inspection of the FT-IR spectra observed for **1b** and **1c** it was noted that the signal for the carbonyl stretch (1737 cm^{-1}) for the fluorenylmethyl (Fm)-ester **1b**

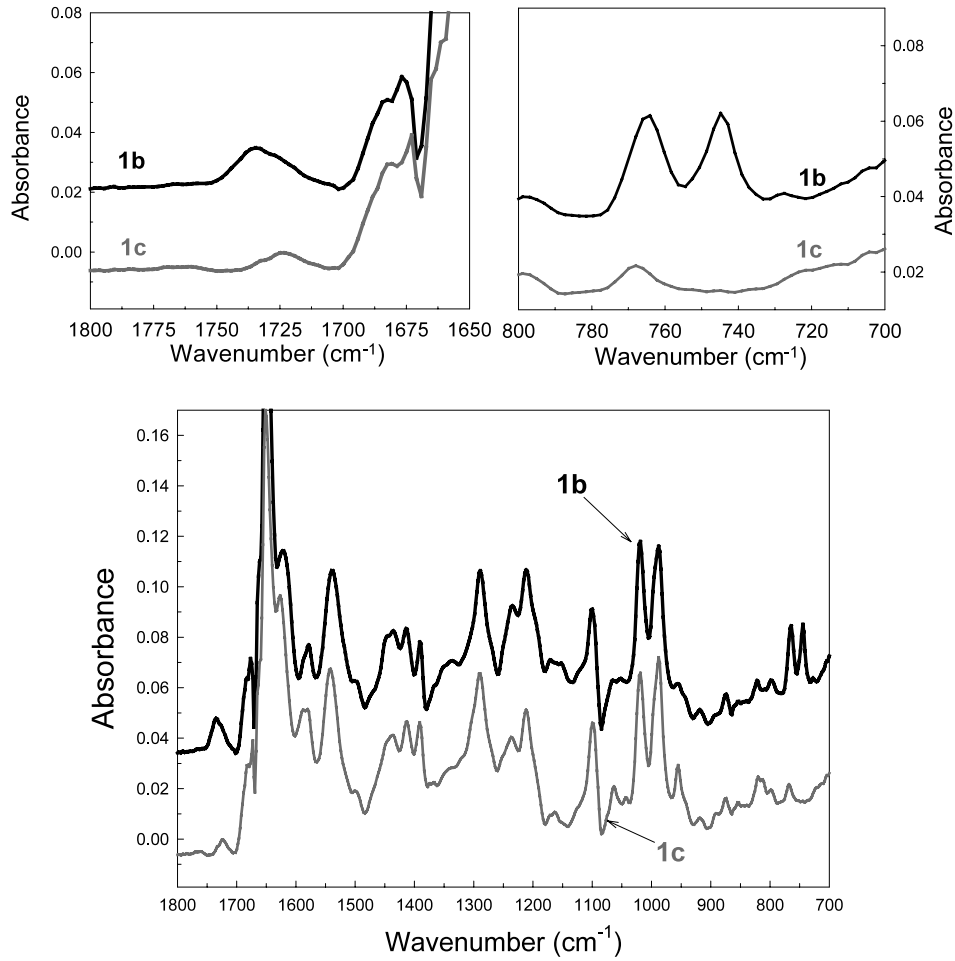
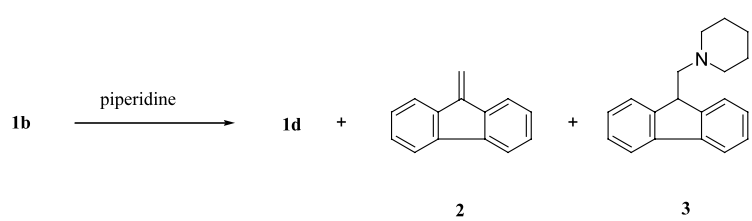


Fig. 1. Solution spectra of **1b** and **1c** after the subtraction of DMF.



Scheme 2.

was well resolved from other carbonyl signals in both the parent and product molecule [16]. Therefore, monitoring the change in intensity of this signal was considered a more reliable means of determining the end point of the reaction. Indeed, this was found to be the case, and a plot of signal intensity (1737 cm^{-1}) against time was found to be in good agreement with the HPLC assay (Fig. 3). Consequently, we then exploited this relationship to develop a calibration model for the reaction.

3.2. Calibration

For calibration three nearly identical experiments were conducted with the initial concentration of **1b** ~60 mM. Since **1b** was quickly ($t_{1/2} = 15$ min) consumed upon addition of piperidine and that the end-point of reaction was of most interest, samples with **1b** less than 25 mM were considered for calibration purposes. Two experiments were used to build a calibration data set and the third was used as the prediction set. From the calibration set, models by PLS were built and used to predict **1b** in the prediction set. These models were constructed with varying conditions including spectral range, number of PLS factors, and data pretreatment process. Many algorithms are available to correlate spectral information to the concentration of an analyte of interest [17]. Both a simple linear regression and factor based multivariate regression were explored for model construction. Simple linear regression could not account for most of the spectral variations related to **1b** because of the huge absorption bands of DMF and the relatively low molecular concentration of **1b**. Therefore, a multivariate regression method, PLS, was applied. Two crucial parameters in the PLS regression process are the spectral range and the number of PLS factors [18]. Based on the absorption features in Fig. 1, we investigated several ranges 1256–1090, 1552–1442, 1780–1703 and 1400–1800 cm^{-1}, including their combinations, where the first three spectral regions differ significantly between **1b** and **1c**. The calibration models were then tested, by employing various combinations of spectral range and different amount of factors. For each combination of parameters (Table 1), the number of factors listed corresponds to the factors recommended by the PLS-1 program based on the *F*-test of the prediction residual error sum of squares (PRESS) which is related to the SECV. It was found that the FT-IR methods built with five PLS factors in the absorption region containing absorption bands at 1737 cm^{-1} and the region 1551–1441 cm^{-1} per-

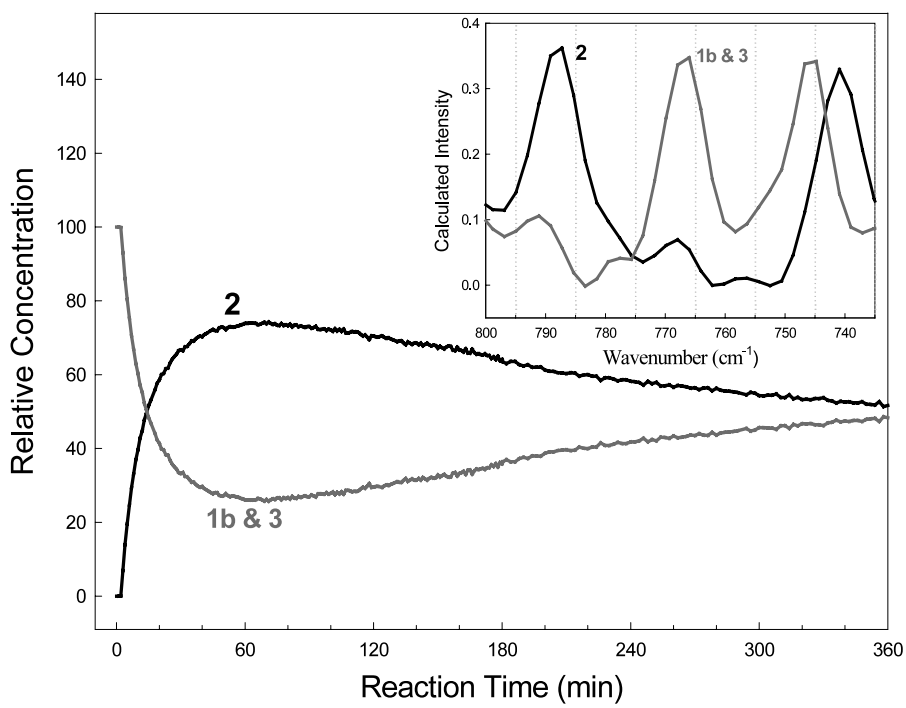


Fig. 2. Profiles of the aromatic C–H out of plane bending for **1b** and **3** compared against that for **2**, acquired by deconvoluting the reaction spectra round 745 cm^{-1}. Inserts are their corresponding component spectra.

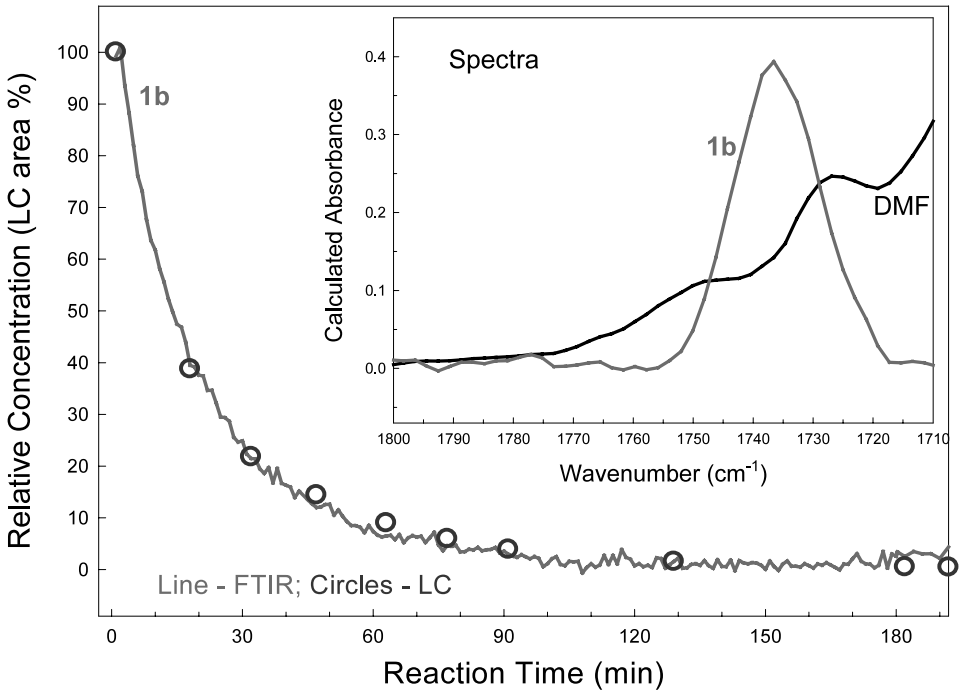


Fig. 3. Profile of **1b** obtained by deconvoluting the reaction spectra around 1735 cm^{-1}, overlaid with LC results (area%). Insert is its corresponding component spectrum.

formed best with a SECV 0.61 mM. Fig. 4 illustrates the performance of this optimum calibration model. In this plot, the concentration correlation between the **1b** levels by HPLC method and FT-IR method is illustrated. Regression analysis (R^2 0.991) results in a slope of 0.998 and a y-intercept of 0.023 mM which demonstrates the closeness of these two methods. When this model was used to predict the concentration of **1b** in the reaction we observed a concentration profile of **1b** that closely matched the HPLC assay values (Fig. 5). Corresponding linear regression (Fig. 5 insert) reveals a coefficient R^2 of 0.9992, a slope of 0.9889 and a y-intercept of -0.1325. A SEP of 0.51 mM was also obtained. This error of prediction is approximately 0.8% of the initial concentration of **1b** and is well within our specifications of $<2\%$ initial concentration.

4. Conclusion

We have shown that FT-IR spectroscopy can be successfully employed to monitor in situ both the progress and end-point of the deprotection of **1b**, $<2\%$ initial concentration of **1b**, a key step,

Table 1
Results from calibration models of **1b** built by PLS regression with different amount of factors in different spectral regions

Spectral range (nm)	Optimum number of PLS factors	SEP (mM)
1780–1703	3	3.43
1551–1441	6	0.73
1256–1090	8	9.83
1800–1400	17	0.59
1780–1703 and 1551–1441	5	0.51
1780–1703 and 1256–1090	5	4.03
1551–1441 and 1256–1090	7	3.58
1780–1703 and 1551–1441 and 1256–1090	5	2.99

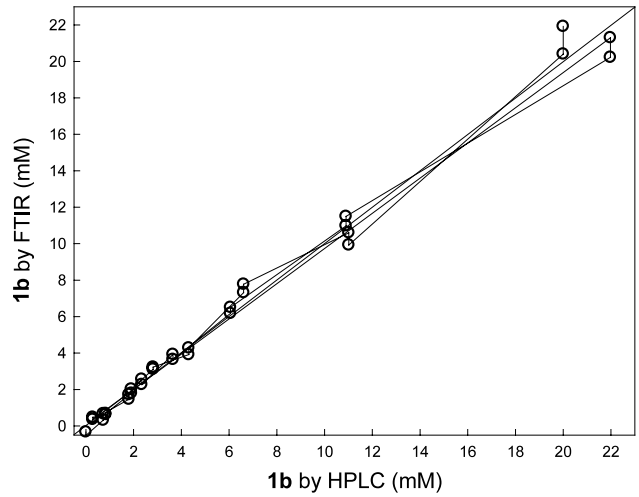


Fig. 4. Concentration correlation plot of **1b** for calibration data set by PLS with five factors in the spectral range of 1780–1703 and 1551–1441 cm^{-1}.

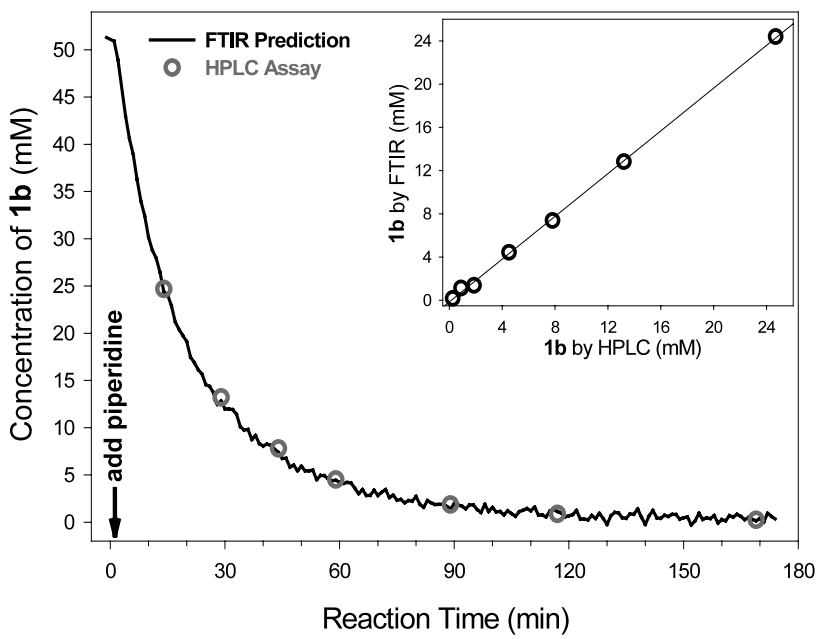


Fig. 5. Concentration profile of **1b** obtained by predicting the prediction run with the calibration model built from five PLS factors in the spectral range of 1780–1703 and 1551–1441 cm^{-1}. Insert is the corresponding correlation plot of **1b** for prediction data set predicted by the same calibration model.

in the synthesis of a pharmaceutically important prostate drug candidate. Real time analysis of this step is critical in minimizing time sensitive by-product formation that may otherwise occur when employing the more conventional time consuming HPLC assay. The FT-IR method requires no sample preparation of the highly toxic reaction mass and consequently, has a significant advantage over conventional chromatographic techniques such as HPLC, TLC and GC that require sample handling and preparation.

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