N-(2-FURYL) ACRYLOYL PENICILLIN: A NOVEL COMPOUND FOR THE SPECTROPHOTOMETRIC ASSAY OF β-LACTAMASE I

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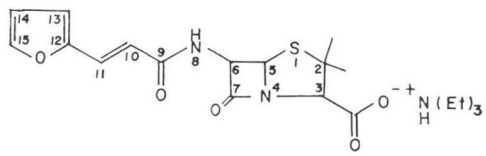
β-Lactamase, capable of hydrolysing the β-lactam ring of either penicillins or cephalosporins have attracted considerable attention in recent years[1~4]. These enzymes are produced by gram-positive as well as gram-negative micro-organisms and are in many instances responsible for the resistance of microbes to β-lactam antibiotics. A variety of procedures for the assay of β-lactamase activity have been developed. These include: (1) iodometric procedures[5~7]; (2) measurement of the H^+ ions released upon hydrolysis of the β-lactam ring either by titrimetric[8] or indicator methods[9]; and (3) spectrophotometric analysis[10,11]. The earliest developed iodometric procedures provide a qualitative rather than a quantitative means for measurement of β-lactamase activity during the various stages of isolation. Furthermore, the possibility of partial inactivation due to modification of an essential tyrosine residue of the enzyme[12] by the I_2 present in the assay medium cannot be completely eliminated.

Recent experiments conducted in our laboratory indicate that iodine, in the absence of substrate, has the ability to inactivate β-lactamase I. Whether these results can be extended to the iodometric assay mixture is not clear at this time. While the titration of the H^+ ions generated during the enzyme catalysed hydrolysis of penicillins offers a convenient and quantitative means of estimating β-lactamase activity, the method requires the somewhat elaborate setup of a pH stat. In addition the incomplete dissociation of the penicilloic acid formed would render the assay less sensitive at pH's below 5.5. The spectrophotometric methods are based on the observed difference in the spectral features between penicillin and that of its penicilloic acid derivative. Thus, the maximum difference in A_{232} nm ($\Delta\varepsilon_m$ of approximately 900 M^{-1} cm^{-1}) between benzylpenicillin and its penicilloic acid derivative has been exploited in the development of an assay procedure for β-lactamase I[10,11]. While this procedure provides a simple and rapid means of assessing enzymatic activity, the need to perform experiments at 232 nm restricts the utility of the procedure especially at high concentrations of substrate (>2 mM) or in buffer media containing species (*i.e.* citrate or acetate) which absorb significantly in this region. In addition more sensitive instrumentation is required for activity determinations at such low wavelengths.

Although a chromophoric substrate, 3-(2,4-dinitrostyryl)-(6R,7R)-7-(2-thienylacetamido)-ceph-3-ene-4-carboxylic acid, has been developed for the measurement of cephalosporinase (β-lactamase II) activity[13], it has been found resistant to β-lactamase I. The advantages of N-(2-furyl) acryloyl substrates in the elucidation of the mechanism of the reaction catalysed by serine proteases has been well documented[14~17]. In light of these considerations, the possibility of N-(2-furyl) acryloyl penicillin as a chromophoric substrate for β-lactamase I was explored. In this communication we wish to report the synthesis of this penicillin and the various advantages it provides in the assessment of β-lactamase activity.

Synthesis of N-(2-Furyl) Acryloyl Penicillin (6-β-Furylacryloylamido-penicillanic acid triethylamine salt)



An ice-cold solution of furylacrylic acid (0.217 g; 1.57 mmoles) and triethylamine (0.026 ml) in dry acetone (13 ml) was treated with ethylchloroformate (0.15 ml; 1.57 mmoles) and stored at 0°C for 5 minutes. The solution was cooled at −50°C and a solution of 6-aminopenicillanic acid (0.339 g; 1.57 mmoles) in 3% aqueous sodium bicarbonate (13 ml) was added. The mixture was stored for 30 minutes at 0°C and then at room temperature for 30 minutes. The mixture was extracted with ether and the aqueous phase adjusted to pH 2.0 with aqueous HCl. The acidic

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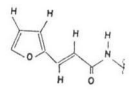
phase was extracted with ether. The dried ether extracts were treated with an excess of anhydrous triethylamine and the solution taken to dryness on a rotary evaporator. The residue was recrystallized from methylene chloride-carbon tetrachloride to give N-(2-furyl) acryloyl penicillin triethylamine salt as pale yellow plates (0.247 g).

Furylacryloyl penicillin triethylamine salt was found to decompose at 107~110°C. The ultraviolet spectrum showed a maximum at 308 nm with an ε_{max} of 20,300 $M^{-1}cm^{-1}$; n.m.r. ($CDCl_3$; Varian T60 spectrometer) δ 1.27 (t; 9H; $\underset{+}{H}N$–(CH_2–CH_3)$_3$), 1.63 (s; 3H; C_2–CH_3), 1.70 (s; 3H; C_2–CH_3), 3.06 (q; 6H; J=7Hz; $\underset{+}{H}N$–(CH_2–CH_3)$_3$), 4.35 (s; 1H; C_3–H), 5.4~5.88 (m; 2H; C_5–H and C_6–H), 6.2~7.6 (m; 7H; H–$\underset{+}{N}$–(Et)$_3$) and



Furylacryloyl penicillin is readily hydrolysed by β-lactamase I, isolated from *Bacillus cereus* 569/H[18]. The difference spectra between furylacryloyl penicillin and its penicilloic acid derivative is illustrated in Fig. 1. It is evident that the hydrolysis of the β-lactam ring is accompanied by changes in absorbance, the maximum being a diminution noted at 330 nm. The $\Delta\varepsilon_m$ values observed at different wavelengths are recorded in Table 1 and these are considerably larger than the $\Delta\varepsilon_m$ value at 232 nm for benzylpenicillin and its penicilloic acid derivative[11]. The data presented permits choice of a wavelength appropriate to the substrate concentrations and spectrophoto-

Fig. 1. Difference spectrum of N-(2-furyl) acryloyl penicillin and the product of enzymatic hydrolysis. A solution of 2.10×10^{-4} M furylacryloyl penicillin in 50 mM phosphate buffer (pH 7.5) was added to two 0.5-cm cuvettes. Following the completion of hydrolysis, initiated by the addition of a 2 μl aliquot of a 3.30 mg/ml enzyme stock solution to the reference cell, the difference spectrum was recorded.

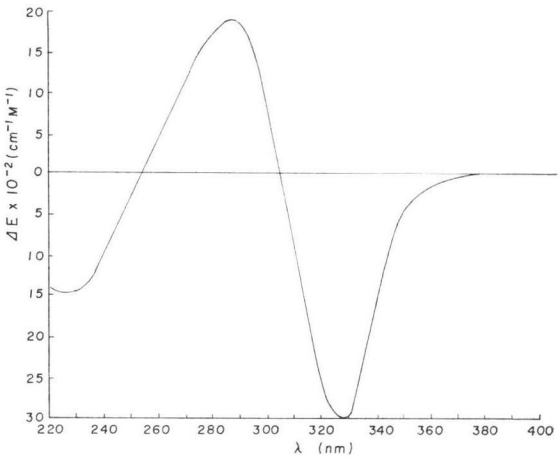


Table 1. Spectral features of furylacryloyl penicillin as a function of pH and wavelength

pH	ε_m substrate (M^{-1} cm^{-1})*			$\Delta\varepsilon_m$ (M^{-1} cm^{-1})**		
	330 nm	340 nm	345 nm	330 nm	340 nm	345 nm
7.5	9504	3458	1632	3048	1771	962
6.5				3015	1701	920
6.0				2917	1674	906
5.5				1999	1116	613
4.5				1278	753	418

ε_m and $\Delta\varepsilon_m$ were measured using a Cary Model 14 spectrophotometer. Extinction values at various wavelengths were calculated from absorbances of the N-(2-furyl) acryloyl penicillin (3.58×10^{-4} M) using appropriate buffers as controls. For the evaluation of $\Delta\varepsilon_m$'s, 2.5 ml of the above penicillin solutions, in appropriate buffers (in cuvettes of 1-cm pathlength), treated with β-lactamase I (10 μl of 0.358 mg enzyme/ml) and 10 μl water served as sample and control respectively.

* ε_m values for substrate were found to be independent of pH over the range shown.

** ε_m values of the product (penicilloic acid derivative) were found to increase significantly below pH 6.0. Consequently, this accounts for the observed drop in $\Delta\varepsilon_m$ values at low pH values. ε_m values could be reversibly shifted upon changes in pH, implying that a simple protonation-deprotonation rather than irreversible intramolecular rearrangements of the product is responsible for the observed phenomenon.

meter used in a particular study. Thus, the rate of hydrolysis of furylacryloyl penicillin can be readily monitored by following the changes in absorbance between 330 and 345 nm with no interference from either the substrate or the buffer medium. The rate of hydrolysis of this chromophoric substrate is linear almost to the completion of the reaction. Neither the substrate nor the product of the reaction are found to be inhibitory. As well, furylacryloyl penicillin proves to be at least as stable as benzylpenicillin in neutral aqueous solution at room temperature. The calculated kinetic parameters, Km (35 μM) and k_{cat} (1.17×10^4 sec^{-1}) are comparable to those observed with benzylpenicillin as substrate (Km, 70 μM; k_{cat}, 2.24×10^4 sec^{-1}). Finally, furylacryloyl penicillin appears to be a class A substrate[19] in that it does not induce conformational changes leading to a less active form of the enzyme.

Considering the above features and the ease of synthesis, furylacryloyl penicillin serves as an excellent chromophoric substrate for the rapid and reliable assessment of β-lactamase I activity.

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