Synthesis, purification and kinetic properties of fluorescein-labelled penicillins

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The synthesis and properties of six fluorescein-labelled penicillins are reported. The two isomers of fluoresceyl-glycyl-6-aminopenicillanic acid are probably the best compounds to use for detection of all the penicillin-binding proteins (PBPs) present in a bacterial membrane preparation. However, the derivatives of ampicillin were much more efficient against *Enterobacter aero-*

genes PBP3. The two isomers obtained when a commercial mixture of the two isomers of carboxyfluorescein was used most often exhibited similar properties, but the *Streptomyces* R61 extracellular DD-peptidase was only efficiently acylated by the 5'-carboxyfluorescein derivative of glycyl-6-aminopenicillanic acid.

INTRODUCTION

The membranes of bacteria contain multiple enzymes involved in the last stages of peptidoglycan biosynthesis that can be irreversibly inhibited by β -lactam antibiotics and are thus directly responsible for the sensitivity of bacteria to these antibiotics (Frère and Joris, 1985).

The study of the membrane-bound penicillin-binding proteins (PBPs) represents the simplest way to assess the role of various PBPs in processes such as cell division and elongation (Spratt and Pardee, 1975) or in the intrinsic resistance mechanisms of various bacteria such as *Streptococcus*, *Enterococcus* or *Pseudomonas*, pathogens of great importance (Bellido et al., 1990; Laible et al., 1991; Piras et al., 1990).

Until recently the most widely used technique was that described by Spratt (1977) more than 15 years ago in which radiolabelled penicillins were utilized. However, this method is time consuming and the detection of the PBPs can take several days or weeks. Recently, a new method was proposed, making use of fluorescent antibiotics. It allowed the detection of less than 10 fmol of PBPs (Galleni et al., 1993). In that earlier study, the fluorescent moiety of the fluorescent penicillins prepared was a mixture of 5' and 6' isomers. Although no major differences between the kinetic properties of these isomers were expected, it seemed safer and more rigorous to prepare the pure compounds. The synthesis, purification and kinetic properties of various pure fluorescent penicillin derivatives are now described in detail.

MATERIALS AND METHODS

Bacterial strain and growth conditions

Enterobacter aerogenes 008-4, a previously described β -lactamase-deficient strain (Curtis et al., 1986), was a gift from N. A. Curtis (ICI Pharmaceuticals, Cheshire, U.K.). Cells were grown in Isosensitest medium (Oxoid, U.K.) at 37 °C with orbital shaking (300 rev./min).

Antibiotics

6-Aminopenicillanic acid (6-APA) was from Beecham (Hep-

pignies, Belgium) and ampicillin from BristolMyers-Squibb (Brussels, Belgium).

Soluble enzymes

The soluble form of *Streptococcus pneumoniae* PBP2x and the extracellular DD-peptidases of *Actinomadura* R39 and *Streptomyces* R61 were purified as described previously by Jamin et al. (1993a), Frère et al. (1974) and Hadonou et al. (1992) respectively.

Preparation of cell envelopes

Membranes were prepared as described by Lindstrom et al. (1970) with some modifications. Briefly, cells were harvested by centrifugation when the culture had reached an A_{600} value of 0.8, resuspended in 30 mM Tris/HCl, pH 8, containing 27% saccharose, 5 mM EDTA and 0.5 mg/ml lysozyme and incubated at 4 °C for 20 min. Cells and spheroplasts were centrifuged for 15 min at 30000 g and resuspended in 10 mM Tris/HCl, pH 8, containing 10 mM MgCl₂ and 20 μ g/ml DNAase to allow lysis of the spheroplasts. Intact cells were removed by centrifugation for 5 min at 5000 g and membranes collected by a 45 min centrifugation at 30000 g. Membranes were washed twice in 50 mM sodium phosphate, pH 7, and stored at a final protein concentration of 20–25 mg/ml in the same buffer at –20 °C.

SDS/PAGE and quantification

Fluorescent-penicillin-labelled PBPs of *E. aerogenes* 008-4 were separated on the ALF DNA sequencer as described by Galleni et al. (1993). Electrophoretic conditions were 400 V, 40 mA and 22 W. For quantification, PBP1 of *Bacillus licheniformis* 749/P22 was selectively labelled and stored in the denaturing buffer, thus providing an internal standard.

Soluble PBPs were loaded on a 10% gel (9 cm $\times$ 7 cm $\times$ 0.075 cm) and electrophoresis was performed for 1 h at 150 V (15 mA). Visualization and band quantification were carried out under u.v. light (λ_{max} , 312 nm) with a two-dimensional densitometer (Cybertech CS-1, Dalton, Waalwijk, The Netherlands).

Table 1 H.p.l.c. conditions for the separation of the various derivatives of carboxyfluorescein

CF and HsF represent respectively the two isomers of carboxyfluorescein and the *N*-hydroxysuccinimide ester of carboxyfluorescein. The detection wavelength was 490 nm.

Derivative	Buffer	Concn. of acetonitrile in elution solvent (% v/v)	Retention times (min)
Flu-6-APA	50 mM sodium acetate, pH 5 50 mM sodium phosphate, pH 7	18 (isocratic) 0–15 min, 9; 15–30 min, 9–13 (linear gradient)	CF: 9.5–13.2; HsF: > 40; Flu-6-APA: 21.2–33.7 CF: 3.4–7.5; HsF: > 30; Flu-6-APA: 11.4–30
Flu-Gly-6-APA	50 mM sodium acetate, pH 5 50 mM sodium phosphate, pH 7	18 (isocratic) 0–15 min, 9; 15–30 min, 9–13 (linear gradient)	CF: 9.5–13.2; HsF: > 40; Flu-Gly-6-APA: 14.5–21 CF: 3.4–7.5; HsF: > 30; Flu-Gly-6-APA: 12.8–20
Flu-ampicillin	50 mM sodium acetate, pH 5 50 mM sodium phosphate, pH 7	30 (isocratic) 16 (isocratic)	CF: 4.1–4.3; HsF: 10.2–16.8; Flu-ampicillin: 7.6–9.2 CF: 2.9–3.3; HsF: 9.1–20.8; Flu-ampicillin: 12.3–30

Determination of the kinetic parameters

The interaction between a β -lactam and a PBP is well represented by the following three-step scheme (Frère et al., 1975a):

where E is the active enzyme, I the β -lactam, EI the Henri-Michaelis complex, EI* the acyl-enzyme and P the degradation product.

Membrane-bound PBPs of *E. aerogenes* 008-4

All the experiments were performed at 37 °C in 50 mM sodium phosphate buffer, pH 7, and the labelled PBPs separated on the ALF DNA sequencer. The two relevant parameters, i.e. k_2/K and k_3 (the acylation and deacylation rate constants), were determined as follows. k_2/K was measured by monitoring the time-dependent accumulation of the acyl-enzyme at fixed β -lactam concentrations as described by eqn. (1):

$$[EI^*]_t/[E]_0 = [kf/(kf + k_3)][1 - e^{-(kf + k_3)t}] \quad (1)$$

where

$$kf = k_2[I]/(K + [I])$$

In practice, the reactions were stopped after various periods of time by addition of the denaturation buffer. The samples were heated at 100 °C for 1 min and subjected to SDS/PAGE. The results were analysed with the help of the ENZFITTER program (Leatherbarrow, 1987). k_3 was determined by measuring the disappearance of the acyl-enzyme as a function of time. PBPs were acylated by a large excess of β -lactam, and a β -lactamase was added to destroy the unreacted antibiotics. Samples were withdrawn after increasing periods of time, the reaction was stopped and the samples treated as above.

Soluble PBPs

Kinetics experiments were performed at 37 °C in 10 mM sodium phosphate, pH 7, or in 100 mM Hepes, pH 8.2, containing 5 mM MgCl₂ for the *Actinomadura* R39 enzyme. The k_2/K values were determined (i) by directly recording the decrease in protein tryptophan fluorescence accompanying the formation of the

acyl-enzyme [*Streptomyces* R61 DD-peptidase and *S. pneumoniae* PBP2x (Nieto et al., 1973; Jamin et al., 1993a)], (ii) by monitoring the decrease in enzyme activity with a reporter substrate [*Streptomyces* R61 and *Actinomadura* R39 DD-peptidases (De Meester et al., 1987)] and (iii) for the three enzymes by direct competition with a β -lactam of known kinetic parameters (Frère et al., 1992).

The k_3 values were determined by monitoring the rate of hydrolysis of the tripeptide substrate Ac₂-L-Lys-D-Ala-D-Ala (Frère et al., 1975b) by the enzyme or as described above for the membrane-bound PBPs.

H.p.l.c. procedures

For analytical purposes, separation was performed on an ET 250/8/4 Nucleosil 5 C18 column (Machery-Nagel) with a flow rate of 1 ml/min. Larger-scale purification was achieved with an ET 250/1/2''/10 Nucleosil 7 C18 column (Machery-Nagel) coupled to a Merck h.p.l.c. apparatus. The flow rate was 4 ml/min and the solvent system was as described in Table 1 and below.

Syntheses

Glycyl-6-aminopenicillanic acid (Gly-6-APA)

N-(4-Nitrobenzyloxycarbonyl)glycine was prepared by allowing glycine (Janssen, Beerse, Belgium) to react with 4-nitrobenzylchloroformate (Fluka, Buchs, Switzerland) as described by Carpenter and Gish (1952). The product was subsequently coupled to 6-APA as described by Doyle et al. (1962). The protective group was removed by hydrogenation (276 kPa) in tetrahydrofuran/water (2:1, v/v) for 18 h at room temperature in the presence of Pd/C (10 %). The mixture was filtered on celite (Sigma, St. Louis, MO, U.S.A.) and washed with the solvent. After evaporation of the organic solvent, the aqueous phase was washed twice with ethyl acetate and freeze-dried. The ¹H-n.m.r. spectrum confirmed the expected structure: δ (p.p.m.) (²H₂O): 1.45–1.56 (6H, 2s, Me₂), 3.58 (2H, s, CH₂), 4.17 (1H, s, H3), 5.51 (2H, H5/H6).

N-Hydroxysuccinimide ester of carboxyfluorescein

Preparation of the activated ester of carboxyfluorescein was performed as follows: a 1:1 isomeric mixture of carboxy-

fluorescein (0.66 mmol; 249 mg; Fluka) was dissolved in 6 ml of dimethylformamide and the solution cooled to 4 °C. Then *N*-hydroxysuccinimide (0.73 mmol; 84 mg; Janssen) and dicyclohexylcarbodi-imide (0.8 mmol; 165 mg; Janssen) were added and the reaction was allowed to proceed at 4 °C for 24 h and the supernatant isolated. The yield was in excess of 95 % as estimated by h.p.l.c. (for conditions, see Table 1).

Synthesis of fluorescent β -lactam compounds

Sodium ampicillin or 6-APA (1.33 mmol) was dissolved in 6 ml of water or 4 % NaHCO₃ respectively, mixed with the solution of *N*-hydroxysuccinimide-activated carboxyfluorescein obtained above (6 ml; 0.66 mmol) and allowed to stand at room temperature for 4 h. The yields (compared with carboxyfluorescein) were 80 % and 60 % for the ampicillin and 6-APA derivatives respectively, as estimated by h.p.l.c.

Gly-6-APA (1.33 mmol) was dissolved in 3 ml of dimethylformamide by silylation with 4 mmol of 3-trimethylsilyl-2-oxazolidinone (Aldrich, Bornem, Belgium). After 1 h at room temperature, the solution became clear and *N*-hydroxysuccinimide-activated carboxyfluorescein (0.66 mmol) was added. The mixture was kept at room temperature for 30 h (yield 56 %).

Water (50 ml) was then added to the solutions and the mixture acidified to pH 2 with 2 M HCl. The products were extracted with ethyl acetate and back-extracted into a water phase continuously maintained at pH 7 during the process. The aqueous phase was freeze-dried, yielding the crude mixtures of isomers which were subsequently resolved and purified by h.p.l.c.

People interested in any of these compounds are invited to contact the authors.

RESULTS AND DISCUSSION

Identification of the fluorescent β -lactams

The commercially available carboxyfluorescein is a mixture of two isomers (Figure 1) which are difficult to separate by routine chemical methods. However (as shown in the Materials and methods section and Figure 2), the separation of the isomers of the various derivatives of carboxyfluorescein can easily be achieved by h.p.l.c. The addition of a β -lactamase to the reaction mixture induced the disappearance of only two fluorescent peaks, which were identified as the isomers of the fluorescent antibiotics. Similar results were obtained for each of the three pairs of synthetic fluorescent antibiotics.

The six different products were finally characterized by ¹H n.m.r. (Table 2). In all cases the 6'-carboxyfluorescein isomer derivative was eluted first on reversed-phase chromatography.

Properties of the fluorescent β -lactams

The affinities of the six fluorescent β -lactams for various high-molecular-mass PBPs and the two standard DD-peptidases of *Actinomadura* R39 and *Streptomyces* R61 are shown in Table 3.

With the three high-molecular-mass PBPs of *E. aerogenes*, the six compounds exhibited very similar acylation rates (k_2/K values in Table 3), which were not significantly different from those observed with benzylpenicillin. The values obtained with this standard antibiotic were very similar to those published for the corresponding proteins of *E. coli* (Curtis et al., 1979). *S. pneumoniae* PBP2x also reacted rapidly with all the compounds. The k_2/K values were significantly lower than those observed with benzylpenicillin and ampicillin but were of the same order of magnitude as or larger than those determined with other

classical compounds such as ticarcillin, temocillin, cephalexin and cefoxitin (Jamin et al., 1993a,b).

All the compounds reacted rapidly with the *Actinomadura* R39 DD-peptidase but the presence of the bulky fluorescent side chain on the amino group of ampicillin decreased its efficiency by a factor of at least 40, as a k_2/K of 70000 M⁻¹·s⁻¹ was measured at 20 °C for intact ampicillin. The *Streptomyces* R61 enzyme exhibited a more specific behaviour. Direct acylation of the amino group of 6-APA or ampicillin yielded nearly inactive compounds. This was, however, to be expected, as, with this enzyme, bulky acyl side chains directly linked to the amino group of 6-APA yield poor inactivators [for instance, quinacillin does not inactivate the enzyme; $k_2/K < 0.1$ M⁻¹·s⁻¹ (Kelly et al., 1981)], and acetylation of the amino group of ampicillin, which is itself a rather inefficient inactivator, also further impairs its acylating properties (Varetto et al., 1987). Conversely, 5'-Flu-Gly-6-APA was an excellent inactivator of the *Streptomyces* R61 enzyme. In fact, of the compounds studied here, only benzylpenicillin exhibited a higher k_2/K value. But surprisingly, the 6'-isomer was two orders of magnitude less efficient. This highly specific effect in a position quite removed from the β -lactam ring itself was rather unexpected. It is hoped that an explanation might be apparent when a refined three-dimensional structure of the enzyme becomes available.

Finally, no high k_3 value was observed, which reflected the reasonable stabilities of the various adducts. The 5.5×10^{-4} s⁻¹

X =

R ¹ -	5'-Flu-6APA
R ² -	6'-Flu-6APA
R ¹ -NH-CH ₂ -CO-	5'-Flu-Gly-6APA
R ² -NH-CH ₂ -CO-	6'-Flu-Gly-6APA
Ph-CH-CO- NH-R ¹	5'-Flu-ampicillin
Ph-CH-CO- NH-R ²	6'-Flu-ampicillin

Figure 1 Structures of the various compounds tested

(a) Chromatogram obtained after a 2 h reaction between the *N*-hydroxysuccinimide ester of carboxyfluorescein and 6-APA. A sample of the reaction medium was diluted 50 times in 50 mM sodium acetate, pH 5, and 20 μ l was injected and eluted on the analytical column under isocratic conditions [50 mM sodium acetate (pH 5)/acetonitrile; 70:30, v/v]. (b) Chromatogram of the same sample after a 15 min incubation with 0.5 μ g of β -lactamase. Note that the compounds eluted at 3.9 min and 4.4 min in (a) have disappeared.

Derivative	Characterization
6'-Flu-Gly-6-APA	δ (p.p.m.) ($^2\text{H}_2\text{O}$): 1.42–1.45 (6H, 2s, Me_2), 4.15 (2H, dd, $-\text{CH}_2-$), 4.22 (1H, s, H3), 5.54 and 5.56, each (1H, J 4 Hz) H5/6, 6.79 (2H, d, J 9.2 Hz, $\text{H}^{2''/7''}$), 6.87 (2H, s, $\text{H}^{4''/5''}$), 7.2 (2H, d, J 9.2 Hz, $\text{H}^{1''/8''}$), 7.7 (1H, s, H^7), 8.05 (1H, d, J 8.1 Hz, H^4), 8.1 (1H, d, J 8.1 Hz, H^5)
5'-Flu-Gly-6-APA	δ (p.p.m.) ($^2\text{H}_2\text{O}$): 1.54–1.62 (6H, 2s, Me_2), 4.28 (2H, s, $-\text{CH}_2-$), 4.3 (1H, s, H3), 5.63 and 5.65, each (1H, J 4 Hz) H5/6, 6.67 (2H, s, $\text{H}^{4''/5''}$), 6.67 (2H, d, J 9.8 Hz, $\text{H}^{2''/7''}$), 7.2 (2H, d, J 9.8 Hz, $\text{H}^{1''/8''}$), 7.5 (1H, d, J 8 Hz, H^7), 8.1 (1H, d, J 8 Hz, H^6), 8.29 (1H, s, H^4)
6'-Flu-ampicillin	δ (p.p.m.) ($^2\text{H}_2\text{O}$): 1.51–1.54 (6H, 2s, Me_2), 4.3 (1H, s, H3), 5.56 (1H, s, CH-Ph), 5.53 and 5.63, each (1H, J 3.8 Hz) H5/6, 6.64 (2H, d, J 9.2 Hz, $\text{H}^{2''/7''}$), 6.76 (2H, s, $\text{H}^{4''/5''}$), 7.01 (2H, d, J 9.2 Hz, $\text{H}^{1''/8''}$), 7.44 (1H, s, H^7) 7.4–7.48 (5H, M , C_6H_5), 7.99 (1H, d, J 9 Hz, H^4), 8.03 (1H, d, J 9 Hz, H^5)
5'-Flu-ampicillin	δ (p.p.m.) ($^2\text{H}_2\text{O}$): 1.62–1.66 (6H, 2s, Me_2), 4.41 (1H, s, H3), 5.71 and 5.73, each (1H, J 3.9 Hz) H5/6, 5.93 (1H, s, CH-Ph), 6.79 (2H, s, $\text{H}^{4''/5''}$), 6.8 (2H, d, J 9.8 Hz, $\text{H}^{2''/7''}$), 7.3 (2H, d, J 9.8 Hz, $\text{H}^{1''/8''}$), 7.52 (1H, d, J 8 Hz, H^7), 7.66–7.76 (5H, M , C_6H_5), 8.16 (1H, d, J 8 Hz, H^6), 8.4 (1H, s, H^4)
6'-Flu-6-APA	δ (p.p.m.) ($^2\text{H}_2\text{O}$): 1.52–1.61 (6H, 2s, Me_2), 4.52 (1H, s, H3), 5.62 and 5.65, each (1H, J 3.8 Hz) H5/6, 6.68 (2H, d, J 9 Hz, $\text{H}^{2''/7''}$), 6.73 (2H, s, $\text{H}^{4''/5''}$), 7.17 (2H, d, J 9 Hz, $\text{H}^{1''/8''}$), 7.64 (1H, s, H^7), 7.94 (1H, d, J 8.1 Hz, H^4), 8.03 (1H, d, J 8.1 Hz, H^5)
5'-Flu-6-APA	δ (p.p.m.) ($^2\text{H}_2\text{O}$): 1.60–1.65 (6H, 2s, Me_2), 4.38 (1H, s, H3), 5.52 and 5.54, each (1H, J 3.9 Hz) H5/6, 6.65 (2H, d, J 9.8 Hz, $\text{H}^{2''/7''}$), 6.68 (2H, s, $\text{H}^{4''/5''}$), 7.23 (2H, d, J 9.8 Hz, $\text{H}^{1''/8''}$), 7.48 (1H, d, J 7.9 Hz, H^7), 8.07 (1H, d, J 7.9 Hz, H^6), 8.27 (1H, s, H^4)

Table 3 Kinetic characteristics of the interaction between the fluorescent β -lactams and some representative PBPs

	<i>E. aerogenes</i> PBP1a			<i>E. aerogenes</i> PBP1b			<i>E. aerogenes</i> PBP2			<i>E. aerogenes</i> PBP3*			<i>Streptomyces</i> R61			<i>Actinomadura</i> R39			<i>S. pneumoniae</i> PBP2x		
	k_2/K (M ⁻¹ .s ⁻¹)	k_3 (s ⁻¹)		k_2/K (M ⁻¹ .s ⁻¹)	k_3 (s ⁻¹)		k_2/K (M ⁻¹ .s ⁻¹)	k_3 (s ⁻¹)		k_2/K (M ⁻¹ .s ⁻¹)	k_3 (s ⁻¹)		k_2/K (M ⁻¹ .s ⁻¹)	k_3 (s ⁻¹)		k_2/K (M ⁻¹ .s ⁻¹)	k_3 (s ⁻¹)		k_2/K (M ⁻¹ .s ⁻¹)	k_3 (s ⁻¹)	
5'-Flu-Gly-6-APA	1100 ± 150	6 × 10 ⁻⁵		550 ± 100	4 × 10 ⁻⁵		250 ± 50	10 ⁻⁴		< 20†	ND		6300 ± 200‡	5 × 10 ⁻⁵		70 000 ± 5000§	8 × 10 ⁻⁶		1600 ± 200	4 × 10 ⁻⁵	
5'-Flu-Gly-6-APA	1200 ± 150	7 × 10 ⁻⁵		250 ± 60	3 × 10 ⁻⁵		400 ± 100	2 × 10 ⁻⁴		< 20†	ND		75 ± 15	5.5 × 10 ⁻⁴		285 000 ± 20 000	3 × 10 ⁻⁵		2100 ± 550	4 × 10 ⁻⁵	
5'-Flu-ampicillin	1400 ± 200	3 × 10 ⁻⁴		400 ± 50	4 × 10 ⁻⁵		1400 ± 150	1 × 10 ⁻⁴		1900 ± 300	1.5 × 10 ⁻⁵		< 1	ND		2000 ± 400‡	5 × 10 ⁻⁶		7000 ± 500§	1 × 10 ⁻⁵	
5'-Flu-ampicillin	1000 ± 200	3 × 10 ⁻⁵		400 ± 100	10 ⁻⁶		1400 ± 200	2 × 10 ⁻⁴		> 9000	2 × 10 ⁻⁵		< 1	ND		3000 ± 400‡	9 × 10 ⁻⁶		2500 ± 300§	2 × 10 ⁻⁵	
5'-Flu-6-APA	ND	ND		ND	ND		ND	ND		ND	ND		< 1	ND		44 000 ± 3000§	2 × 10 ⁻⁵		400 ± 45	7 × 10 ⁻⁵	
5'-Flu-6-APA	ND	ND		ND	ND		ND	ND		ND	ND		< 1	ND		55 000 ± 5000§	4 × 10 ⁻⁵		860 ± 90	6 × 10 ⁻⁵	
Benzylpenicillin	> 1000	10 ⁻⁴		250 ± 25	5 × 10 ⁻⁵		600 ± 100	1.5 × 10 ⁻⁴		2800 ± 600	10 ⁻⁴		13 000	1.4 × 10 ⁻⁴		300 000	3 × 10 ⁻⁶		58 000	5.5 × 10 ⁻⁵	

ND, not done.
* Appears as a doublet.
† < 30% labelling after 30 min of incubation with 10 μM fluorescent antibiotic.
‡ Determined by the reporter substrate method.
§ Determined by time-dependent decrease in enzyme fluorescence.
|| Determined by competition with an antibiotic of known kinetic parameters.

ND, not done.

* Appears as a doublet.

+ < 30% labelling after 30 min of incubation with 10 μ M fluorescent antibiotic.

† Determined by the reporter substrate method.

§ Determined by time-dependent decrease in enzyme fluorescence.

§ Determined by time dependent decrease in chlorzime fluorescence.

k_3 value observed in the interaction of 6'-Flu-Gly-6-APA with the *Streptomyces* R61 enzyme still translates into a half-life of 20 min. With *E. aerogenes* PBP2, the k_3 values were around 10^{-4} s^{-1} , not significantly different from that obtained with benzylpenicillin.

In conclusion, these fluorescent compounds supply a practical alternative to the radioactively labelled antibiotics for the detection of PBPs. As shown previously (Galleni et al., 1993), about 0.2 pmol of any given PBP can be detected with the naked eye, and, with the help of an ALF DNA sequencer, the sensitivity increases to about 5–10 fmol.

In preliminary experiments (not shown), the highly resistant PBP5 of *Enterococcus hirae*, which is similar to the *Staphylococcus aureus* PBP2', could also be labelled with the mixture of Flu-ampicillins and Flu-Gly-6-APA.

The isomer mixture might be satisfactory for detection of all the PBPs present in a bacterial membrane preparation. An accurate determination of the kinetic parameters, however, requires utilization of the purified single isomers.

This work was supported in part by the Belgian government in the form of the 'Poles d'attraction interuniversitaire' (PAI no 19), an 'Action concertée' with the Belgian government (convention 89/94-130), the 'Fonds de la recherche scientifique médicale' (contract no. 3.4537.130) and a 'convention tripartite' between the Region Wallonne, SmithKline Beecham, U.K., and the University of Liège. B.J. and J.M.B. are 'Chercheurs qualifiés' and M.J. is 'Chargé de recherche' of the FNRS (Brussels, Belgium).

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