# Labelling of penicillin-binding proteins from *Escherichia coli* with photoreactive derivatives of β-lactam antibiotics

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## 1. INTRODUCTION

β-Lactam antibiotics exert their bactericidal effect by interacting with a number of enzymic proteins integrated in the bacterial membrane (review [1]). Within these proteins, usually known as penicillin-binding proteins (PBPs), those catalysing the last steps in peptidoglycan synthesis are of particular interest, since their enzymic activities (transpeptidase and DD-carboxypeptidase) are inhibited as a consequence of their interaction with the β-lactam antibiotics. PBPs from a number of bacteria have been characterized on the basis that they form a covalent bond with the β-lactam antibiotics [2]. This bond can be stabilized by thermal denaturation of the β-lactam–PBP intermediate complex in the presence of sodium dodecyl sulfate (SDS) [2,3]. Membranes containing PBPs labelled with a radioactive β-lactam can be fractionated on SDS–polyacrylamide gel electrophoresis and the PBPs are recognized in the gel by conventional fluorographic methods. Thus, the PBPs known and studied so far have been detected by using the few radiolabelled β-lactams that are available: benzyl[$^{14}$C]penicillin; [$^{3}$H]benzylpenicillin; and either $^{125}$I- or $^{3}$H-labelled derivatives of ampicillin [2–5]. PBP patterns depend on the labelled antibiotic used, and hence doubts still remain as to whether other PBPs might exist to which the radioactive β-lactams used so far do not appear to bind by the standard methods used due to their low affinity. To overcome these problems we have synthesized a number of photoreactive radiolabelled β-lactams that react and form permanent covalent bonds with the PBPs, since photoreactive ligand derivatives have been used to some extent for structural studies on membranes and other biological structures (reviews [6,8]). Chemical and photochemical labelling of a receptor by its ligand are important techniques to elucidate the nature of the ligand–receptor interaction, and for identification and characterization of receptors. We have synthesized two β-lactam derivatives each containing two different photoreactive moieties. One of them is an aryl azido compound, widely known as a photoreactive reagent for labelling studies [6,8], whereas the other one contains a nitroguaiacol derived group used in photochemical studies with other biological materials [9].

## 2. EXPERIMENTAL

*Escherichia coli* W 7(10) was used throughout this work. Cells were grown in rich liquid medium [11] at 37°C under forced aeration and harvested in the mid-log phase. Cell envelopes were prepared as described previously [12]. Unless otherwise stated, all binding studies were done in the dark by using *E. coli* membranes (1.0 mg protein/ml) suspended in 50 mM Na borate buffer (pH 8.5). The membranes were incubated with increasing concentrations of radioactive β-lactams at 37°C

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for 15 min. Reaction was stopped by rapid cooling of the samples and immediate centrifugation at 0°C for 10 min at 30000 × *g*. Pelleted membranes were resuspended in a small volume of 50 mM sodium phosphate buffer (pH 7.0) and proteins were fractionated by SDS–PAGE polyacrylamide gel electrophoresis following essentially the method in [2]. Slab gels were prepared for fluorography as in [13], dried under vacuum and exposed on prefogged X-Omat Kodak X-ray films. Exposure times ranged from 7–20 days. For photolabelling studies, 1 ml *E. coli* membrane suspension (1.0 mg protein/ml) was incubated with the β-lactam for 15 min at 37°C. The membrane suspension, contained in a 1-cm quartz UV cell, was subjected to irradiation under monochromatic light from a LH 151N 1000 W Xenon lamp and a GM252 grating monochromator, slit width 5.9 nm (Schoefeld Instruments, Westwood NY). The distance of the sample from the light source was 105 cm. The sample, at 20°C, was stirred frequently during irradiation time to compensate the inner filter effect. Samples with the aryl azido derivative were irradiated at 270 nm for ⩽ 20 min, while those of the nitroguaiacol β-lactam were irradiated at 340 nm for up to 180 min. Control samples were maintained in the dark for the same periods of time under identical conditions. Membranes were then cooled down and centrifuged at 30000 × *g*, for 10 min at 0°C. Precipitates were then resuspended in ice-cold 50 mM sodium phosphate buffer (pH 7.0). The centrifugation was repeated 4 times and the final pellet resuspended in a small volume of the phosphate buffer and electrophoresed as above. Earlier, unbound β-lactam was removed by repeated centrifugation of the membranes before illumination. Although this procedure reduced to some extent the background of the fluorographic plates, it proved to be unnecessary and was subsequently omitted. When the azido β-lactam derivative was used, some samples, after illumination, were subjected to treatment with 0.5 M neutral hydroxylamine and the mixture was incubated at 37°C for 30 min. Samples were further treated as described above.

Proteins were estimated by the Folin phenol reagent method using bovine serum albumin as a standard. Benzyl[$^{14}$C]penicillin (51 Ci/mol), [$^{3}$H]acetic anhydride (9 Ci/mmol) and sodium boro[$^{3}$H]hydride (20 Ci/mmol) were purchased from Amersham International (Amersham, Bucks). 6-Aminopenicillanic acid and amoxycillin were gifts from Antibióticos SA (Madrid). Other chemicals were reagent grade from either Sigma Chemical Co. (St Louis MO) or Merck (Darmstadt).

## 3. RESULTS

### 3.1. *Synthesis of the photoreactive β-lactams*

The procedure for the synthesis of the photoreactive β-lactams is shown in fig.1. Purity of all products and intermediates used was assessed by thin-layer chromatography on silica gel. Furthermore, infrared and NMR spectra of the products were in agreement with the proposed structures. Compound (I) was readily obtained from equimolecular amounts of 4-nitroguaiacol (potassium salt) and potassium chloroacetate, both in dimethylformamide solution and incubated for 3–4 h at 100°C. The carboxylic group of (I) was condensed with the amino group of the β-lactam amoxycillin to give (II) via the hydroxysuccinimide ester, following the conventional method in [14]. Our β-lactam (II) was radioactively labelled by incubation with a little more than a double molar amount of [$^{3}$H]acetic anhydride for 16 h at room temperature in 50 mM Na-borate buffer (pH 8.5) to give compound (III). This procedure produced full acetylation of the hydroxyl group of the β-lactam and total hydrolysis of the excess of anhydride. For the preparation of the azido derivative, 4-azidosalicylaldehyde was obtained essentially as in [15] but the reduction of azidosalicylic acid imidazolide was done with AlLi tri-*t*-butoxyhydride instead of using AlLi hydride. This procedure increased the yield of the aldehyde up to 40%. Treatment of 4-azidosalicylaldehyde with potassium chloroacetate in basic medium gave (IV) which was then coupled with 6-aminopenicillanic acid as above [14], yielding (V). Final reduction of the aldehyde compound was obtained by incubation with an excess of sodium boro[$^{3}$H]hydride at 0°C for 30 min obtaining compound (VI).

### 3.2. *Affinity of the photoreactive β-lactams for penicillin-binding proteins*

When binding experiments were performed in the dark, the two photoreactive β-lactam com-

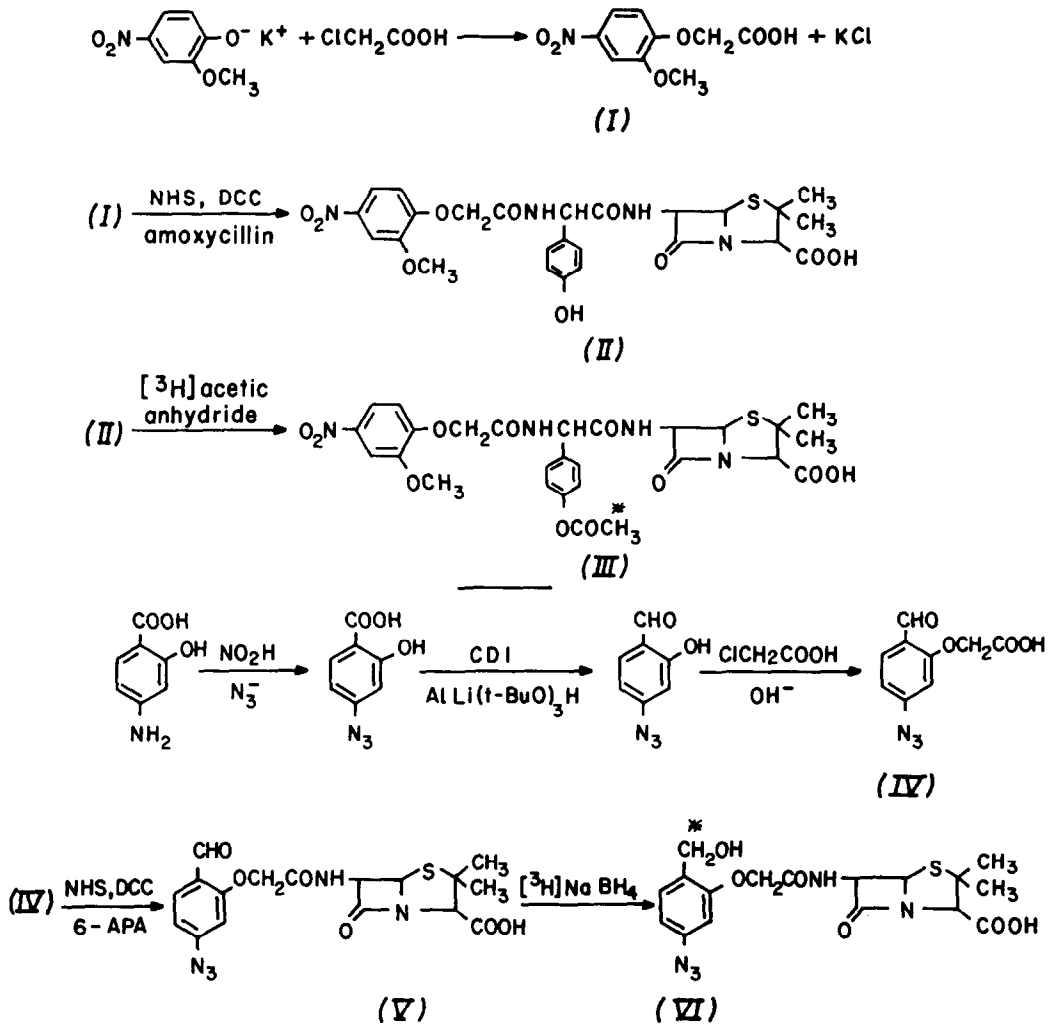


Fig.1. Pathway followed for the synthesis of our $\beta$-lactam photoreactive derivatives. *Abbreviations:* NHS, *N*-Hydroxysuccinimide; DCC, dicyclohexylcarbodiimide; CDI, carbonyldiimidazole; (*) location of the radioatom.

pounds (VI) and (III) displayed affinity for all PBPs labelled when benzyl[$^{14}$C]penicillin is used (fig.2). Quantitative data showing binding affinities of the two $\beta$-lactams are presented in table 1. The nitroguaiacol derivative displayed, in general, higher affinities for the PBPs than the parental $\beta$-lactam amoxycillin. The only difference observed from the standard PBP pattern (elicited in fig.3 but not in fig.2) consisted of the appearance of a new $\beta$-lactam-binding protein of $M_r$ 170000. The existence of this protein will be discussed later.

### 3.3. *Photolabelling of the penicillin-binding proteins*

Previous experiments demonstrated that irradiation with the monochromatic light used did not affect the electrophoretic pattern of membrane proteins as stained by Coomassie blue. PBPs from irradiated membranes resulted unaffected in their electrophoretic mobilities and also in their capacities to bind benzyl[$^{14}$C]penicillin (fig.3A,B) by irradiating the membranes at 270 nm for 20 min, the conditions chosen as most suitable. Indeed labelling with compound (VI) increased with

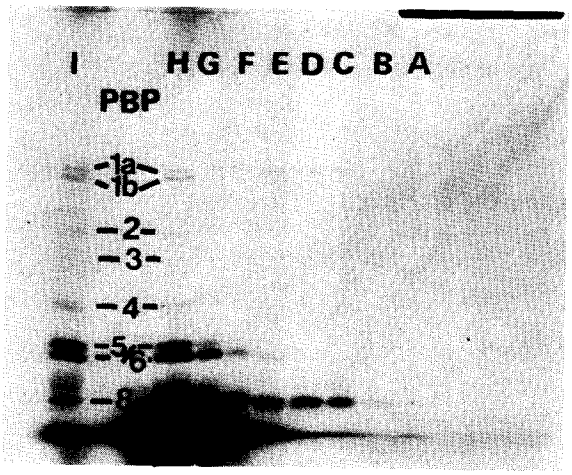


Fig.2. Polyacrylamide gel electrophoresis showing the binding of compound (VI) to PBPs from *E. coli* membranes. Experiments done in the dark under the conditions in section 2. Concentrations of compound (VI) ($\mu$g/ml) were: (A) 0.03; (B) 0.09; (C) 0.27; (D) 0.82; (E) 2.5; (F) 7.4; (G) 22.2; (H) 66.6. Benzyl[$^{14}$C]penicillin was used in lane (I) at 20 $\mu$M.

time up to 30 min, but significant labelling was obtained after 1 min irradiation. Fig.3 also shows the extent of labelling obtained with compound (VI). Lanes (C,E) correspond to non-illuminated samples while (D,F) were irradiated for 20 min. Samples (C,D) were subjected to hydroxylamine treatment. This nucleophilic agent is known to produce breakage of the benzylpenicillin–PBP intermediates to some extent [2]. By comparing fig.3(C) and (E) most of the label disappeared due to hydroxylamine treatment, although PBPs 1a and 1b were still present in (C). These results agree with those reported for benzylpenicillin [2]. We can conclude from fig.3 that:

(i) A $M_r$ 170000–175000 protein became labelled either in irradiated or in non-irradiated samples;

(ii) The $\beta$-lactam was removed from this protein with hydroxylamine;

(iii) The $M_r$ 170000–175000 protein that also appeared, though unsteadily, when membranes were labelled with benzyl[$^{14}$C]penicillin, can be considered a specific $\beta$-lactam-binding protein.

We have further shown that the $M_r$ 170000–175000 protein was also labelled with other radioactive $\beta$-lactams, including several *bis*-$\beta$-lactam antibiotics used as breakable crosslinkers and this radioactive band did not correspond to any undissociated PBP of lower $M_r$ (unpublished). The finding that hydroxylamine could remove the antibiotic from this high-$M_r$ PBP suggests that the protein binds to the $\beta$-lactam through a nucleophilic attack, just as other PBPs do [2]. Furthermore, a band corresponding to an $M_r$ 190000 protein appeared upon illumination and disappeared with hydroxylamine treatment, whereas inverse behaviour was seen in PBP 1a which disappeared upon illumination and appeared with hydroxylamine. Similar behavior was observed with the $M_r$ 105000 protein and PBP 3. These findings can be explained on the basis of a probable photocrosslinking of two molecules of PBP 1a (to give a band of $M_r$ 190000) and PBP 3 (giving a band of $M_r$ 105000). Indeed, using *bis*-$\beta$-lactam antibiotics we have obtained dimeric forms of PBP

Table 1

Affinity of photoactive $\beta$-lactams for the penicillin-binding proteins (PBPs) from *Escherichia coli* membranes ($ID_{50}$)[a]

| $\beta$-Lactam | 1a | 1b | 1c | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|---|
| Compound (III) | 0.5 | 0.5 | 0.2 | 0.1 | <0.04 | 5 | 20 | 3 |
| Amoxycillin[b] | 4 | 20 | 2 | 2 | 1 | 0.2 | >200 | 20 |
| Compound (VI) | 0.25 | 65 | 60 | 20 | 20 | 0.25 | 20 | 5 |

[a] $ID_{50}$ is the concentration of $\beta$-lactam ($\mu$g/ml) that produced half-saturation of the PBPs

[b] Affinities of amoxycillin are given to compare them with the derivative compound (III). These data with amoxycillin were kindly given to us by Dr M.A. de Pedro (personal communication)

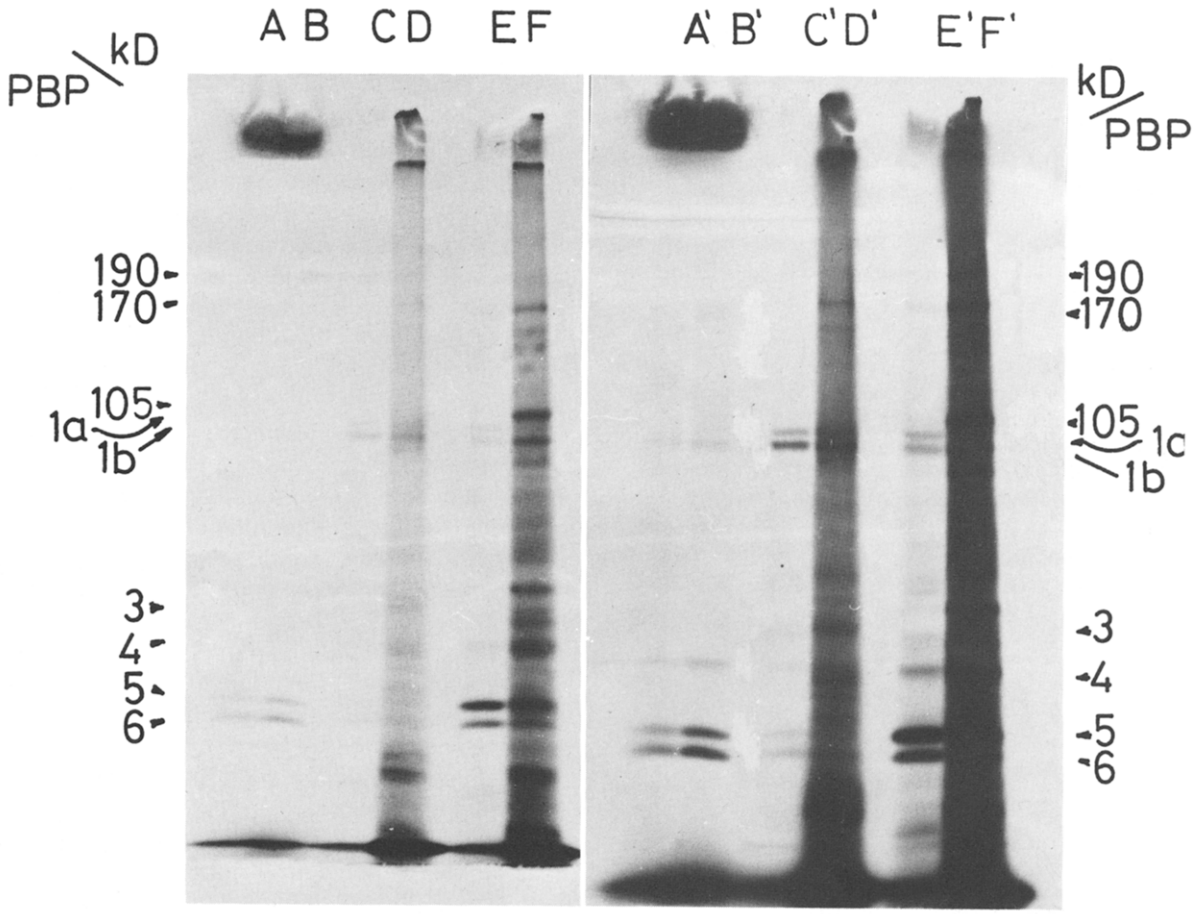


Fig.3. Polyacrylamide gel electrophoresis showing the photolabelling of PBPs from *E. coli* membranes by compound (VI). The experimental procedure was as in section 2. Specific conditions were: (A), (A′) membranes incubated for 20 min at 20°C in the dark then mixed and incubated with 20 $\mu$M benzyl[$^{14}$C]penicillin; (B), (B′) membranes irradiated for 20 min at 270 nm then incubated with 20 $\mu$M benzyl[$^{14}$C]penicillin; (C), (C′) and (E), (E′) membranes mixed with 100 $\mu$g/ml of compound (VI) then incubated for 20 min in the dark; (D), (D′) and (F), (F′) membranes incubated with 100 $\mu$g/ml compound (VI) then irradiated at 270 nm for 20 min; (C), (C′) and (D), (D′) membranes treated with 0.5 M neutral hydroxylamine after the binding with compound (VI); (D), (D′) and (F), (F′) membranes were not treated with hydroxylamine; (A–F) 7 days exposure; A′–F′ 20 days exposure.

3 corresponding to $M_r$ 105000 (unpublished). PBP 2 and, even more, PBP 3 are very sensitive to our photolabelling procedure, while PBPs 5 and 6 behaved in the opposite way, as hydroxylamine easily removed the labelling. Two new bands located between PBP 4 and PBP 5 appeared in the irradiated samples and the label was not removed by hydroxylamine treatment, suggesting that the $\beta$-lactams bound to the proteins through the photoreactive azido group.

The $M_r$ 170000 protein also appeared with compound (III) but disappeared when the irradiation time was extended (fig.4). PBPs 1a and 4 were extralabelled upon irradiation while PBPs 1b and 3 disappeared as other protein bands derived from them ($M_r$ 145000 and 110000, respectively) were elicited. Two new bands located between PBP 4 and PBP 5 were also detected in these experiments (fig.4).

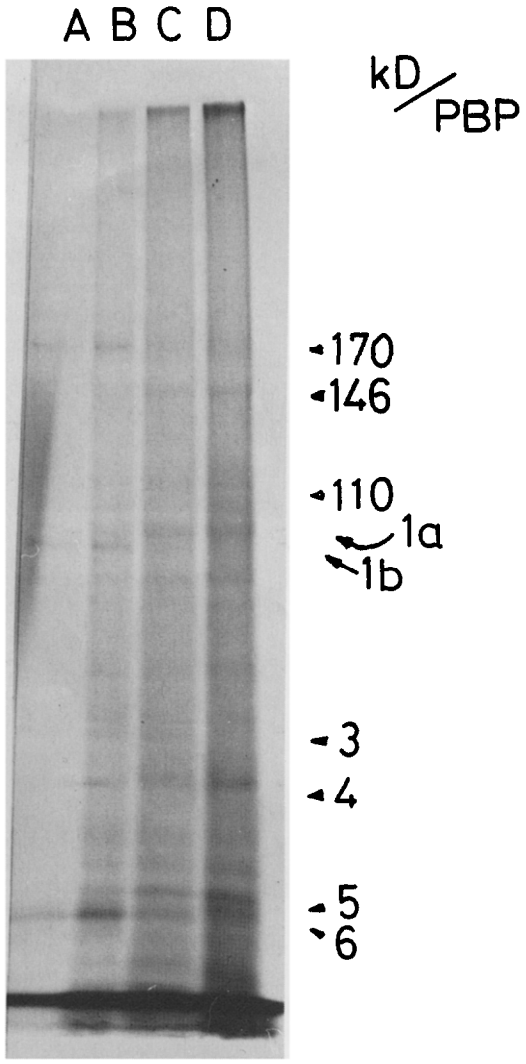


Fig.4. Polyacrylamide gel electrophoresis showing the photolabelling of PBPs from *E. coli* membranes by compound (III). Membranes were incubated in the dark with 50 $\mu$g/ml compound (III) and then subjected to irradiation for the following periods: (A) no irradiation (time 0 min); (B) 30 min; (C) 90 min; (D) 180 min.

## 4. DISCUSSION

All known PBPs of *E. coli* membranes that have been reported with benzylpenicillin are also detected with the radioactive $\beta$-lactams synthesized and used here, suggesting the specificity of the interactions observed in our compound. Moreover, our new $\beta$-lactam derivatives also labelled an $M_r$ 170000 protein. Data presented and discussed suggest that this $M_r$ 170000 protein is a PBP undescribed before and not an aggregation of the smaller PBPs.

Nearly all PBPs were extralabelled by our $\beta$-lactams (VI) and (III) when the samples were irradiated. In the case of the azido $\beta$-lactam (compound (V)) considerable differences were detected between the hydroxylamine treated and untreated samples. As hydroxylamine destroys the covalent bond between the $\beta$-lactam and the protein, our results clearly show that illumination produced an extra covalent bond between the PBPs and the photoreactive groups of the $\beta$-lactams. However, intermolecular photoreactions between $\beta$-lactams and proteins appear to be negligible if there is not really any type of interaction between the molecules without illumination. Similar electrophoretic patterns, from the qualitative point of view, were seen, whether free $\beta$-lactams were washed out, or not, before illumination. Since our photoreactive $\beta$-lactams photolabelled PBPs at 76 $\mu$M compound (III) and 222 $\mu$M compound (VI), it is most likely that the interactions of these $\beta$-lactams with the protein targets prior to illumination, are required to facilitate the intermolecular photoreaction.

Our compounds (III) and (VI) appear to cause cross-linking between membrane proteins to a certain extent. We do not know precisely the distance between the reactive groups of our $\beta$-lactams but according to the extended models, these distances are of 12 Å and 14 Å in compounds (III) and (VI), respectively. It is most interesting that compound (VI) cross-links two molecules of either PBP 1a, giving an $M_r$ 190000 band or PBP 3, giving a new band of $M_r$ 105000, whereas compound (III) cross-links two molecules of either PBP 1b or PBP 3. In both cases, the monomer PBPs decreased and even disappeared upon irradiation. This observation is in agreement with the appearance of higher $M_r$ bands after illumination.

The appearance of a number of new radioactively labelled bands, mainly between PBP 4 and PBP 5, after membrane treatment with compound (III) and (VI) shows that membrane proteins other than PBPs also interact with these $\beta$-lactams. Therefore the use of photoactivable radioactive $\beta$-lactams as protein affinity labels and cross-linkers can be very useful to elucidate the topology of a number of proteins in the membrane.

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