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ESTER PRODRUGS OF AMPICILLIN TAILORED FOR INTRACELLULAR ACCUMULATION

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Abstract: Seven new ester prodrugs of ampicillin with hydrolysis half-lives ranging from 65 to 308 min were synthesized. The cellular accumulation of two of them (in J774 mouse macrophages) and their activities against intracellular *Staphylococcus aureus* were determined in comparison with the pivaloyloxymethylester of ampicillin (pivampicillin) and ampicillin. The esters accumulated extensively and were more active than ampicillin in this *in vitro* system. © 1997 Elsevier Science Ltd.

The successful treatment of intracellular bacterial infection requires the use of antibiotics capable of accumulating to sufficient an extent in the host cells [i.e. above the minimal inhibitory concentration (MIC) of the drug]. As reviewed earlier[1,2], macrolides, fluoroquinolones and ansamycines have this property, but their use is often limited by constraints related to spectrum, toxicological profiles and emergence of resistance affecting most if not all derivatives within each class.

In this context ß-lactam antibiotics could represent an useful alternative because of their usually excellent tolerance, their potentially very broad spectrum, and the availability of derivatives active against strains resistant to early compounds. Yet, ß-lactams are unable to act against several types of intracellular bacteria[3-7], mostly because they do not accumulate in the host cells[1,2].

Using penicillin G as a model, we demonstrated that the masking of its carboxylate function by the formation of an aminopropylamide derivative, N-(3-dimethylaminopropyl)-[^{14}C]benzylpenicillinamide (pKa around 10) allows the corresponding derivative to accumulate in cells (at levels about 5 times the extracellular concentrations) and to localize partly in lysosomes[8], in accordance with the well known property of weak organic bases to accumulate in acidic, membrane-bounded subcellular organelles[9]. Carboxamides, however, are not prodrugs of β-lactam antibiotics since the amide bond is not easily cleaved[10], and this derivative of penicillin G remains therefore microbiologically inactive[8].

We have now applied the concept of basic prodrugs of ß-lactams to ampicillin, since this molecule already carries the necessary basic function on its side chain, allowing us more freedom in the design of a reversible masking of the carboxylate. In our strategy, simple esters (e. g., methyl esters) were discarded since the β-lactam ring is more rapidly cleaved than this ester function[11]. In contrast, pivampicillin **1** (Figure1), and all double esters of ß-lactam antibiotics are known to be extremely sensitive to chemical and enzymatic hydrolysis[12]. We reasoned that a better stability could be reached if the pivaloyl ester function of **1**, a possible site of attack by

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nucleophiles, was removed. We thus synthesized esters **2**-**8** (Figure 1). The aim of the structural variations in these esters was to cover a range of sensitivities to hydrolysis by tuning the electron-withdrawing power (compounds **2**-**7**) and size (compound **8**) of the substituents.

To test the activity of our compounds, we relied on a model of intracellular *S. aureus* infection in which macrolides and fluoroquinolones, but not ß-lactams are active (because of the lack of accumulation)[13], and in which the organism is most presumably located in acidic endosomes and phagolysosomes[14].

The compounds were synthesized by alkylation of the carboxylate function of ampicillin, the key point being the temporary protection of its amino function by *in situ* formation of a benzaldimine[15]. The synthons used to obtain **2**-**7** were described in the literature[16]. The strategy used to synthesize **8** is sketched in Figure 2.

Hydrolytic stability of the esters: the rates of hydrolysis of esters **1**-**8** in phosphate buffer, pH 7.4, were determined by HPLC. The observed half-lives are reported in Table 1. The hydrolysis of all compounds was base-catalyzed. The effect of pH on the apparent first-order rate constant is illustrated in Figure 3 for compound **7**. Ampicillin was a major product. As ampicillin esters have *per se* no bactericidal activity, the minimum inhibitory concentrations (MIC) values against *S. aureus* after hydrolysis is a good indication of the amount of ampicillin released[17]. The MIC of **1**, **7** and **8**, after hydrolysis 24 h in phosphate buffer, were 0.65 µM, 0.53 µM, and 1.16 µM, respectively, while ampicillin featured a MIC of 0.5 µM after standing 24 h in the same buffer (the slowly-hydrolysing ester **8** released less ampicillin probably because the rate of the β-lactam ring opening became similar to that of the ester hydrolysis[18]). A detailed analysis of the side-products formed was not undertaken. Contrary to **1**, the half life of which decreased in 80 % human or fetal calf serum to 50-55 min. (serum : 0.1 M Na^+/K^+ phosphate buffer in 0.75 M NaCl, 8:2 v/v), the half-life of **7** remained practically unchanged in those conditions. Furthermore, the addition of mouse peritoneal macrophage homogenate (1 mg of protein per ml of buffer) did not modify the half-life of **7**. Compounds **7** and **8** were selected for biological studies, in comparison with **1** and ampicillin because these two monoesters were situated at the lower and upper limits of the stability range.

Accumulation in J 774 macrophages: Jusko's fluorimetric method was used to quantify intracellular ampicillin[19]. This method is based on the acid hydrolysis of ampicillin at 90°C in the presence of formaldehyde to give 2-hydroxy-3-phenyl-6-methylpiperazine[20, 21]. The same compound is obtained from the ester prodrugs or even α-aminobenzylpenicilloic acid, so that ampicillin, its precursors and its decomposition products are titrated together. Figure 4 shows the apparent cellular to extracellular concentration ratio (C_c/C_e) of the prodrugs of ampicillin when J 774 macrophages were incubated with compounds **1**, **7** or **8** (the C_e was 10 µM). [Cells incubated with ampicillin did not accumulate the drug. The apparent C_c/C_e ratio at steady state was around 1 (data not shown)]. Esters **1**, **7** and **8** gave rise to a marked accumulation of *circa* 70, 40, and 40, respectively, after 30 min. These ratios decreased, however with time to reach an equilibrium value of about 1 after 6 h except for **8**. For **1** and **7**, a microbiological assay[22] gave results similar to Jusko's procedure, demonstrating that the bulk of the cell-associated compounds was ampicillin or a precursor of ampicillin (the microbiological assay entails a 4 h or 24 h incubation at 65 or 37°C in a water-based medium so that full conversion of the precursors is most likely to occur during the procedure). For **8** however, the microbiological determination gave a lower value than the chemical method, suggesting that the rate of hydrolysis of the ß-lactam ring of **8** was of the same order of magnitude as that of the ester function. We interpret the data of accumulation and efflux shown for **1** and **7** as indicating that these basic and lipophilic prodrugs first accumulate into cells but progressively regenerated ampicillin both intra- and extracellularly, so that the partition after 24 h is that of ampicillin itself.

Intracellular activities of ampicillin, **1**, **7** and **8** : J 774 macrophages were infected by *S . aureus* [23], to obtain a mean value of 1 viable intracellular bacteria per cell (non-phagocyted bacteria were removed by treatment with lysostaphin). Cells were then incubated with ampicillin or the esters **1**, **7** or **8** at a C_e of 10 times their MIC (see above) for up to 24 h. At selected times, the number of viable bacteria in cells was quantified by colony counting assay[23] [a study made by confocal microscopy (staining of the bacteria with fluorescein isothiocyanate before the infection of the cells, and of the cell actin with phalloidine rhodamine after permeabilization of the membrane) demonstrated that the bacteria remained confined in cells for a period of approx. 10 h, whereas at 24 h both intracellular and extracellular bacteria were present, probably as a result of their partial release from dead cells]. As shown in Figure 5, compounds **1**, **7** and **8** were about five times more active than ampicillin in this system, with no marked difference among them. We interpret this observation as related to the enhanced accumulation of ampicillin through the use of the corresponding prodrugs. Yet, we cannot simply correlate accumulation with activity data since (a) we do not know how much of the prodrug is effectively converted into ampicillin intracellularly; (b) the accumulation of ampicillin, if it occurs, remains transient, so that many bacteria may not have suffered irreversible damage; (c) part of the intracellular bacteria may sojourn in a compartment inacessible to ampicillin and/or its prodrugs.

In spite of these difficulties and uncertainties, the conclusion of this work is that an improved cellular pharmacokinetics of ampicillin by use of basic prodrugs results in an enhancement of the potency against intracellular bacteria. With suitable modifications, this strategy is also in principle applicable to other penicillins and ß-lactams in general, including ß-lactamase-resistant molecules, opening therefore a renewed area of research and development for antibiotics active against intracellular infections.

FIGURE 1

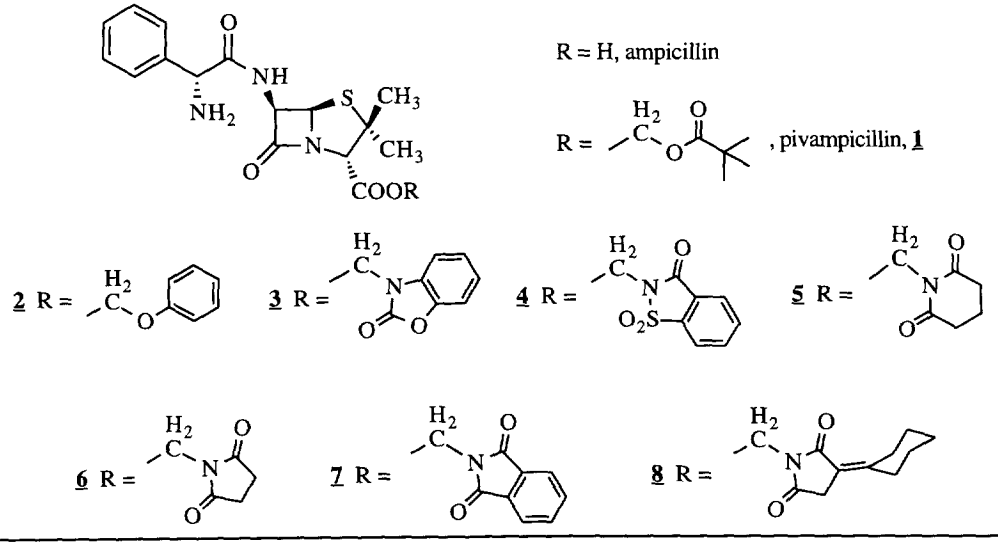
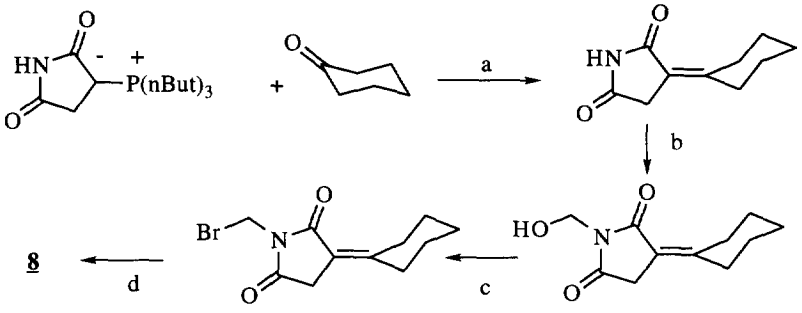


TABLE 1 : HYDROLYTIC STABILITIES OF THE ESTERS

Half-lives (min) in 0.02 M phosphate buffer, 0.15 M NaCl, pH 7.4, 37°C.

1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
120	215	150	65	180	150	65	308

FIGURE 2 : SYNTHESIS OF 8



(a) Phosphorus ylid[24] + neat cyclohexanone (5 molar equivalents), 100° C, 4 h. Yield 20 %.
(b) 20 % formalin, 100° C, 5 min. Yield 82 %.
(c) PBr_3 (1 molar equivalent), toluene, reflux 4 h. Yield 70 %.
(d) same procedure as in note 15.

FIGURE 3

Rate constants of hydrolysis of 7 as a function of pH (k_{app} in min.)

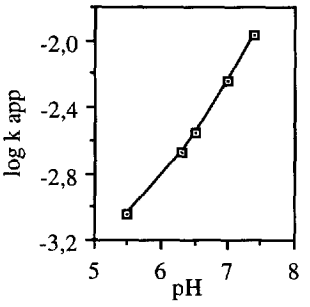


FIGURE 4

C_c/C_e ratio of compounds 1, 7, and 8 as a function of time when J774 macrophages were incubated with these compounds at a concentration of 10 µM in the culture medium. (open circles - plain line : fluorimetric method; plain circles - dotted line : microbiological method)

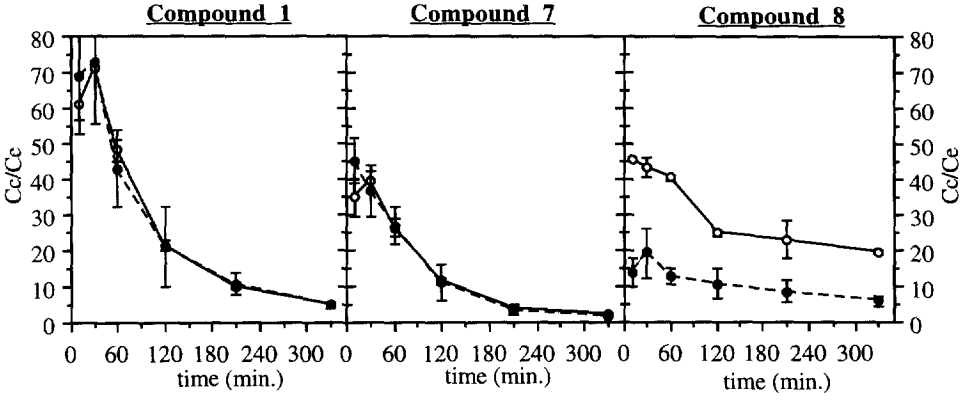
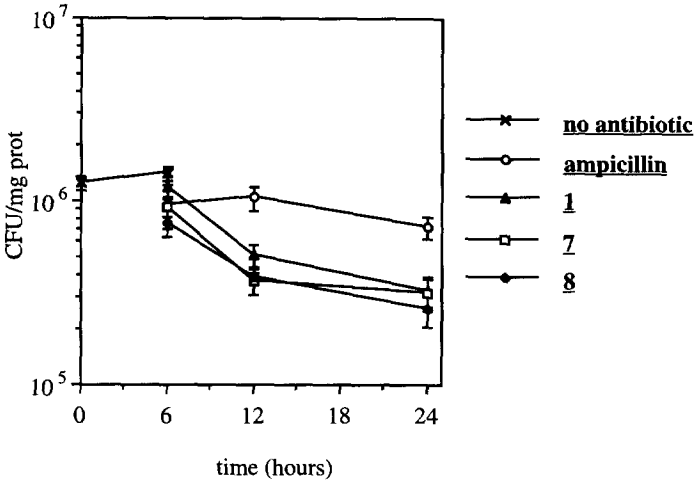


FIGURE 5

Number of *S. aureus* colony forming units per mg of proteins after cellular lysis as a function of time when infected J774 macrophages were incubated without (control) or with compounds **1**, **7**, **8** or **ampicillin** at a concentration equal to 10x their MIC (against *S. aureus*). At 12 h, bacterial counts in controls (no antibiotics) was > 10^7, but infection was both intracellular and extracellular (presumably due to bacteria-induced macrophage death). No extracellular bacteria was noted at 12 and 24 h in the presence of ampicillin or the prodrugs.



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15 **Typical procedure: the synthesis of 7** : a mixture of ampicillin trihydrate (2 g, 5 mM), potassium bicarbonate (0.5 g, 5 mM), benzaldehyde (1 ml, 10 mM) and DMF (50 ml) was stirred at 0° C for 12 h. Anhydrous magnesium sulfate (1.2 g, 10 mM, heated at 800° C for 24 h) was added to trap the water, and, after a few minutes, a further equivalent of potassium bicarbonate (0.5 g, 5 mM) and N-(bromomethyl)phthalimide (1.2 g, 5 mM). After stirring 24 h at 0° C, the mixture was poured into cold water (80 ml) and extracted with ethyl acetate (3 x 60 ml). The organic extract was washed with brine (3 x 50 ml), dried over magnesium sulfate and filtered. The filtrate after evaporation under vacuum was stirred at -15° C for 20 min. in a 1:1 mixture of acetonitrile and water (40 ml) adjusted at pH 2.5 with 0.5 M

HCl. Water was added (40 ml) and the pH was adjusted to 5.0 by adding potassium bicarbonate. This solution was freed of acetonitrile by evaporation under vacuum, washed with ethyl acetate (4 x 50 ml) and saturated with sodium chloride. The formed precipitate was collected by filtration and washed with dichloromethane and ether. Yield 1.9 g, 76 %. m.p. 159-160° C. The IR, ^1H and ^{13}C spectra were in agreement with the structure. Elemental analysis (hydrochloride, monohydrate): Calc. C, 53.33, H, 4.83, N, 9.95, Na, 0.0; Found, C, 53.38, H, 4.94, N, 9.79, Na, 0.045.

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17 The MIC is defined as the lowest concentration of antibiotic giving no visible bacterial growth (naked-eye examination) after a 24 h incubation at 37° C in a conventional growth medium (tryptic soy broth, TSB, pH 7.4).

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19 **Determination of the C_c/C_e ratio for ampicillin, 1, 7 and 8:** J 774 macrophages (Ralph, P., Prichard, J. and Cohn, M. *J. immunol.*, **1982**, *114*, 898) were cultured at 37° C in 95% air-5% CO_2 in RPMI 1640 medium supplemented with 10% calf serum (GIBCO Ltd., Paisley, Scotland). Cells were seeded in petri dishes (diameter, 6 cm) at a density of approx. 10^5 cells per cm^2 and grown 2-3 days until confluency. The compound to be tested was added to reach an external concentration of 10 µM. After incubation with the cells, the medium was aspirated, the cell sheet washed with ice-cold phosphate-buffered saline (4 x), scraped off with a rubber policeman, suspended in ice-cold sterile water (1 ml), and subjected to brief sonication to obtain a homogenous suspension. The protein concentration was determined by the method described by Lowry et al. (Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. *J. Biol. Chem.*, **1951**, *193*, 265). Ampicillin, its prodrugs and its ring-opened derivatives were then quantified as a whole by the fluorimetric method described by Jusko. The C_c was calculated on the consideration that all the material detected was ampicillin, taking into account that 1 mg of cell protein corresponds to a cellular volume of 5 µl[11]. The C_c/C_e ratio was expressed by reference to the content of the extracellular medium in ampicillin (based on its content in pro-drug), .

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22 a 20 or 100 µl sample of the suspended cell was placed on sterile disks upon a plate with *Bacillus Stearothermophilus* ATCC 10149 in PM indicator agar (code 1800 Difco Laboratories, Detroit Mi USA). The zones of bacterial growth inhibition were read after 4 h of incubation at 65°C. This method detected ampicillin from 0.01 µg/ml to 1µg/ml. In the range from 0.1 to 10 µg/ml, *Bacillus subtilis* ATCC 6633 on conventional nutrient agar (24 h, 37°C) was also used.

23 **Intracellular bactericidal activities of ampicillin, 1, 7, and 8**: a suspension of *S. aureus* (10^9 CFU/ml) in RPMI 1640 (4.5 ml) was opsonized by incubation with fresh human serum (0.5 ml) at 37°C for 30 min. The bacteria were centrifugated and resuspended in RPMI 1640 containing 10% of foetal calf serum. The bacterial concentration was determined by turbidimetry, using a calibration curve relating the absorbance to the number of colony forming units per ml (CFU/ml). J 774 macrophages at confluency were infected by an inoculum of two bacteria per cell (37°C, 1 h). The culture was washed with lysostaphin (2 U/ml, 30 min) to selectively kill residual extracellular bacteria. The cells were then incubated for the time indicated in RPMI 1640 + 10% foetal calf serum, pH 7.4, in the presence of ampicillin or 1, 7, 8 at 10 times their MIC. The cells were harvested and treated as described for the C_c/C_e determination. The amount of viable bacteria (CFU/mg of cellular proteins) was determined after spreading on suitable nutrient agar and incubation at 37°C for 20 h. (see for an analog model of infected J774 macrophages : Rastogi, N., Potar, M. C. and David, H. L. *Ann. Inst. Pasteur Microbiol.*, **1988**, *139*, 225).

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