Hydrolysis of Pro-Ala Dipeptides by Lysosomal Hydrolases. Models for the Study of Lysosomotropic Amino Acid Prodrugs of Penicillins

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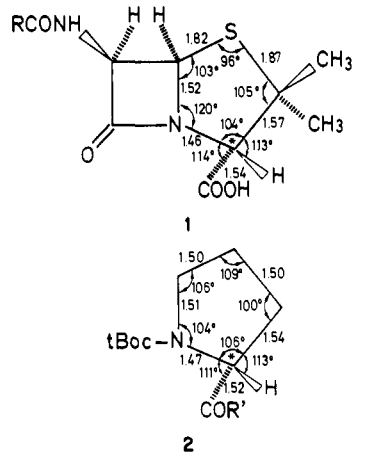
Peptidic lysosomotropic prodrugs of antibiotics and antitumoral agents could be of advantage in chemotherapy, providing that free, active drug is released at, or close to, the desired site of action. Thus, aminoacyl derivatives of doxorubicin, e.g., where the drug is attached to the amino acid by a primary amino function, are sensitive to lysosomal hydrolases. We have examined whether a similar approach can be used for drugs carrying a carboxyl group such as β-lactam antibiotics. Because the C adjacent to the carboxyl group in β-lactams has the D configuration, we have examined and report here the synthesis and susceptibility of model peptides, namely Boc-D-Pro-L-Ala and Boc-L-Pro-L-Ala to lysosomal hydrolases. Hydrolysis of the D isomer proceeds considerably more slowly than that of the L isomer. Lysosomal carboxypeptidase(s) and/or amidases appear therefore to have a much narrower specificity than aminopeptidase(s), which will severely limit the applicability of the concept of peptidic lysosomotropic prodrugs.

The development of prodrugs is an approach to increase the activity of many pharmacological agents by modifying their tissue or subcellular distribution or by allowing their activation at the desired site of action (see ref 1 for review and examples in ref 2–4). In antibacterial chemotherapy, most of the available drugs have little activity against intraphagocytic bacteria[5,6] partly because their intracellular accumulation is either too low or too slow to achieve useful therapeutic levels.[7,8] For β-lactam antibiotics, this lack of intracellular accumulation is probably due to their acidic character, since it is usually observed that weak organic acids are excluded from membrane-bound acidic compartments.[9] This is of particular importance for the lysosomes, the pH of which may be as low as 4–5,[9,10] but also holds true for the cellcytoplasm, which is often 0.2–0.5 pH unit lower than the extracellular environment. Conversely, basic drugs[8,12–15] accumulate in macrophages and other cells. Recently, we observed that a basic derivative of penicillin, namely *N*-[[3-(dimethylamino)propyl]benzyl]-penicillinamide, enters macrophages and accumulates in lysosomes.[16] This derivative is, however, inactive because of the substitution of the free carboxyl group in C_3 of the penicillin (see Chart I), which is essential for antibacterial activity.[17] Moreover, this amide linkage is resistent to lysosomal hydrolases.[16]

Chart I



Structural formulas: (1) penicillins (R is $-CH_2O-$ for penicillin V; asterisk indicates the asymmetric carbon (C3) adjacent to the carboxyl group; (2) Boc-D-Pro-L-Ala (R′ is $-HNCH(CH_3)COOH$; asterisk indicates the asymmetric carbon). The interatomic distances (Å) and bond angles (deg) are shown for both the thiazolidine ring of penicillin and for the proline ring.[20,21]

We therefore thought of intercalating an amino acid or a peptide between the basic moiety and the penicillin, with the hope that it could be cleaved off by lysosomal peptidases. [The (pivaloyloxy)methyl or phthalyl esters already described (e.g., pivampicillin and talampicillin) are not useful in the present case, since they are quickly hydrolyzed outside cells by serum esterases.[18]] It was not clear from the available literature[19] whether lysosomes were endowed with enzyme activities capable of rapidly splitting the proposed linkage between the amino acid and the penicillin molecule. In penicillins, indeed, the C adjacent to the carboxyl group (C_3) has the D configuration. We therefore have first synthesized and report here on the hydrolysis of model peptides, namely Boc-D-Pro-L-Ala [Boc = (*tert*-butyloxy)carbonyl] and its isomer Boc-L-Pro-L-Ala,

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(1) Stella, V. J.; Himmelstein, K J. *J. Med. Chem.* **1980** *23*, 1275.
(2) Carl, P. L.; Chakravarty, P. K.; Katzenellenbogen, J. A.; Weber, M. J. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 2224.
(3) Kingsbury, W. D.; Boehm, J. C.; Mehta, R. J.; Grappel, S. F. *J. Med. Chem.* **1983**. *26*, 1725.
(4) Masquelier, M.; Baurain, R.; Trouet, A. *J. Med. Chem.* **1980** *23*, 1166.
(5) Holmes, B.; Quie, P. G.; Windhorst, D. B.; Pollara, B.; Good, R. A. *Nature*, **1966**, *210*, 1131.
(6) Solberg, C. O. *Acta Med. Scand.* **1972**, *191*, 383.
(7) Lange, A.; Assman, G. *Z. Med. Mikrobiol. Immunol.* **1966** *153*, 60.
(8) Prokesch, R. C.; Hand, W. L. *Antimicrob. Agents Chemother.* **1982**, *21*, 373.
(9) Waddell, W. J.; Bates, R. G. *Physiol. Rev.* **1969**, *49*, 285.
(10) Reijngoud, D. J.; Tager, J. M. *Biochim. Biophys. Acta* **1977**, *472*, 419.
(11) Ohkuma, S.; Poole, B. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 3327.
(12) Wibo, M.; Poole, B. *J. Cell. Biol.* **1974**, *63*, 430.
(13) Johnson, J. D.; Hand, W. L.; Francis, J. B.; King-Thompson, N.; Corwin, R. W. *J. Lab. Clin. Med.* **1980**, *95*, 429.
(14) Noël, G.; Petterson, C.; Trouet, A.; Tulkens, P. *Eur. J. Cancer* **1978**, *14*, 363.
(15) Tulkens, P.; Trouet, A. *Biochem. Pharmacol.* **1978**, *27*, 415.
(16) Renard, C.; Zenebergh, A.; Vanderhaeghe, H. J.; Claes, P. J.; Tulkens, P. M. In *Proceedings of the 14th International Congress on Chemotherapy*, Kyoto, Japan, 1985, in press.
(17) Frère, J. M.; Duez, C.; Duart, J.; Coyette, J.; LeyR-Bouille, M.; Ghuyssen, J. M. In *Enzyme Inhibitors as Drugs*; Sandler, M., Ed.; MacMillan: London, 1980; pp 183–207.
(18) Mandell, G. L.; Sande, M. A. In *The Pharmacological Basis of Therapeutics*; Goodman Gilman, A., Goodman, L. S., Gilman, A., Eds.; MacMillan: London, 1980; pp 1143–1145.
(19) McDonald, J. K.; Schwabe, C. In *Proteinases in Mammalian Cells and Tissues*; Barret, A. J., Ed.; North-Holland: Amsterdam, 1977; pp 311–391.

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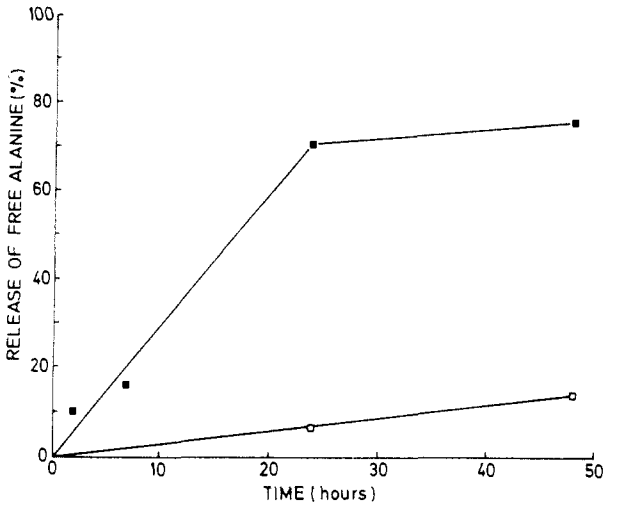

Figure 1. Release of free alanine from Boc-L-Pro-L-Ala (■) and Boc-D-Pro-L-Ala (□) upon incubation in the presence of a soluble extract from liver lysosomes. The ordinate shows the amount of alanine liberated as the percentage of the total amount of alanine present in the dipeptide. The release of free alanine from hydrolysis of lysosomal proteins was less than 5% and has been substracted.

by lysosomal hydrolases. The Boc group is intended to prevent the action of an aminopeptidase, thus leaving the Pro–Ala peptide bond sensitive only to carboxypeptidase (1). As shown in Chart I, the interatomic distances and bond angles of proline and of the thiazolidine part of penicillin are fairly similar, especially with respect to the atoms adjacent to the C_3.[20,21]

Results and Discussion

Thin-layer chromatography showed a distinct release of Ala after even 1 h of incubation of Boc-L-Pro-L-Ala in the presence of lysosomal enzymes. This release markedly increased after 24 h of incubation. Conversely, with Boc-D-Pro-L-Ala as substrate, free Ala became detectable after only 24 h of incubation. No release of Ala was seen in the absence of enzyme preparation. The Ala liberated by autolysis of the proteins of the enzyme preparation contributed to only a small part of the overall amount of Ala detected, as shown in experiments where the dipeptide was omitted.

To check that the liberation of Ala did not proceed from hydrolysis of some deprotected peptide (Pro-Ala) by aminopeptidase, we looked for the appearance of free Pro. None was detected. Conversely, a liberation of Boc-Pro was observed by thin-layer chromatography, in parallel to the liberation of Ala. This indicates that hydrolysis effectively proceeded from the carboxyl end, through the action of a carboxypeptidase.

Figure 1 shows on a quantitative manner the liberation of free Ala from Boc-L-Pro-L-Ala and Boc-D-Pro-L-Ala, over 48 h of incubation with lysosomal extracts, as measured by amino acid analysis. About 70% of the total Ala available was already released from Boc-L-Pro-L-Ala after 24 h, whereas only 7% was liberated from Boc-D-Pro-L-Ala after 24 h and 14% after 48 h. This release represents true hydrolysis of the Boc-D-Pro-L-Ala dipeptide, and not hydrolysis of contaminating Boc-L-Pro-L-Ala since the identity and purity of the amino acids (98%) were checked before synthesis and the conditions used for the synthesis of the dipeptide do not cause racemization.

Thus, our data demonstrate that prolylalanine dipeptides can be cleaved by lysosomal carboxypeptidase(s) but the rate of this reaction is considerably slower when proline has the D configuration. On the other hand, we may exclude hydrolysis by prolinase (EC 3.4.13.8), since this enzyme is only found in the cytosol and requires the imino group of proline to be free.[22]

Although the hydrolysis of Boc-D-Pro-L-Ala dipeptide is not negligible, it is likely that the hydrolysis of penicillin-Ala-COOH, and of derivatives of penicillins with other amino acids, will be too slow to allow efficient intracellular release of free active drug. We indeed examined a number of such derivatives synthesized from penicillin V,[23] but none of them showed significant release of free, active antibiotic in vitro in the presence of lysosomal extracts or of cultured macrophages, or even in vivo after injection to mice (Renard, C.; Tulkens, P. M., unpublished work). The present report may rationalize these observations.

Our results therefore show that the design of lysosomotropic peptidic prodrugs may be fraught with difficulties related to enzyme specificities. Masquelier et al. reported that aminoacyl derivatives of other drugs such as Ala-doxorubicin or Leu-doxorubicin rapidly release free in amino acid and drug upon exposure to lysosomal enzymes.[4] In this case, however, the amino acid was attached by its carboxyl function to an amino group of the drug. Hydrolysis of the prodrug of doxorubicin is probably carried out by aminopeptidase(s) that would therefore appear much less selective than carboxypeptidases.[19] Thus, substituents other than amino acids will need to be used for the successful design of lysosomotropic prodrugs of compounds that only offer a carboxyl group as attachment point.

Experimental Section

Peptide Synthesis and Purification. All amino acids and reagents were of analytical purity. D-Proline was purchased from UCB Bioproducts (Brussels). The dipeptides D-Pro-L-Ala and L-Pro-L-Ala were synthesized by the solid-phase method of Merrifield[24] and purified by chromatography on a silica gel 60 column (Lobar-Merck) with ethanol/water (8:2, v/v) as eluent. They were identified by thin-layer chromatography on silica gel 60 plates (Merck) in the same eluent system. Spots were visualized by chlorine reagent. These peptides were subsequently protected by a (*tert*-butyloxy)carbonyl group[25] (Boc). The purity of these imino-blocked peptides was checked by TLC and amino acid analysis after hydrolysis with 6 N HCl at 110 °C for 22 h.

Peptide Hydrolysis by Lysosomal Extracts. A soluble fraction, isolated from highly purified rat liver lysosomes[26] was used throughout at a protein concentration of 4.6 mg/mL. To 0.5 mL of 100 mM acetate buffer (pH 5) were added 0.25 mL of the lysosomal preparation, 0.1 mL of 50 mM cystein, and 0.15 mL of the peptide, suitably dissolved in water to obtain a 8 mM concentration in the final mixture. Controls were run either without lysosomal extract or without peptides. Incubation was carried out at 37 °C, and aliquots were taken at various times for analysis.

Detection of Hydrolysis Products. Proteins were precipitated by addition of an equal volume of acetonitrile, and the

(20) Mitsui, Y.; Tsuboi, M.; Iitaka, Y. *Acta Crystallogr., Sect. B: Struct. Crystallogr. Cryst. Chem.* **1969**, *25*, 2182.
(21) Sweet, R. M. In *Cephalosporins and Penicillins Chemistry and Biology*; Flynn, E. H., Ed.; Academic: New York, 1972; pp 281–309.
(22) Myara, I.; Charpentier, C.; Lemmonier, A. *Life Sci.* **1984**, *34*, 1985.
(23) Bounkhala, Z. Thesis, Université Catholique de Louvain, Belgium, 1983, p 170.
(24) Merrifield, R. B. In *The Chemistry of Polypeptides*; Katsoyannis, P. G., Ed.; Plenum: New York, 1973; pp 336–361.
(25) Moroder, L.; Hallett, A.; Wuñsch, E.; Keller, O.; Wersin, G. *Hoppe-Seyler's Z. Physiol. Chem.* **1976**, *357*, 1651.
(26) Trouet, A. *Methods Enzymol.* **1974**, *31*, 323.

hydrolysis products were separated by thin-layer chromatography of the supernatants. We used two different eluent systems (ethanol/water (8:2, v/v) for the separation of Ala from Boc-Pro-Ala; chloroform/methanol/acetic acid (85:10:5, v/v) for the separation of Boc-Pro from Boc-Pro-Ala). Ala was revealed by ninhydrin and Boc-Pro or Boc-Pro-Ala by chlorine reagent. In parallel, the release of Ala was quantitatively measured by amino acid analysis using an automatic amino acid analyzer (Beckman Model 120 C).

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Registry No. Boc-D-Pro-L-Ala, 101653-95-6; Boc-L-Pro-L-Ala, 36301-70-9; hydrolase, 9027-41-2; carboxypeptidase, 9031-98-5; amidase, 9012-56-0; penicillin, 61-33-6.

Synthesis and Biological Activity of 5-Phenylselenenyl-Substituted Pyrimidine Nucleosides

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Several 5-phenylselenenyl derivatives of pyrimidine nucleosides were synthesized by electrophilic addition of phenylselenenyl chloride to the nucleosides under basic conditions. With use of this route, 5-(phenylselenenyl)-6-azauracil (6) was also prepared. These compounds may serve as inhibitors of thymidylate synthase, as potential antiviral and anticancer agents, and as versatile intermediates for the synthesis of 5- or 6-substituted nucleosides. 5-(Phenylselenenyl)arabinosyluracil (PSAU, 4) and the corresponding cytosine analogue (PSAC, 5) were poor inhibitors of a promyelocytic leukemia cell line that was arabinosylcytosine-resistant. PSAU and PSAC were significantly less active than *ara*-C against L1210 cells and were found to selectively interfere with the cellular uptake and/or phosphorylation of 2′-deoxycytidine and 2′-deoxyuridine in intact L1210 cells.

Much effort in the development of antiviral and anticancer drugs has been concentrated on the inhibition of enzymes necessary for purine or pyrimidine nucleoside synthesis.[1,2] Most of these compounds exert their effect on replicating cells and viruses either by blocking the salvage or the de novo pathways of nucleotide biosynthesis, nucleic acid biosynthesis, or nucleic acid function after incorporation into the macromolecule. This paper describes the synthesis and biological activity of several new pyrimidine nucleosides substituted at the C-5 position with a phenylselenenyl group.

The rationale for adding a selenium moiety on the C-5 position of pyrimidine nucleosides was twofold. First, some of the uracil derivatives may inactivate thymidylate synthase (EC 2.1.1.45), an enzyme that undergoes conjugate addition with the 5,6-unsaturated portion of pyrimidine nucleotides,[3] by the oxidative mechanism shown in Scheme I. Although at present there is no evidence that oxidation of selenium can take place intracellularly, one can presume that by analogy with sulfur that this process may take place in vivo. For example, the antiinflammatory drug sulindac can undergo oxidation in animals and humans.[4] Further support for this oxidation is provided by the facile preparation at room temperature of the stable 5-(phenylseleninyl)-2′-deoxyuridine by the action of hydrogen peroxide on 5-(phenylselenenyl)-2′-deoxyuridine (Chart I, 3) in tetrahydrofuran (unpublished results). Several other nucleosides containing selenium were previously synthesized and some were found to have biological activity.[5-11] For example, Choi et al.[5] recently found that 5-hydroseleno-2′-deoxyuridylate is a potent inhibitor of *Lactobacillus casei* thymidylate synthase. This compound was synthesized as an analogue of 5-mercapto-2′-deoxyuridylate, a known inhibitor of this enzyme.[12]

Scheme I

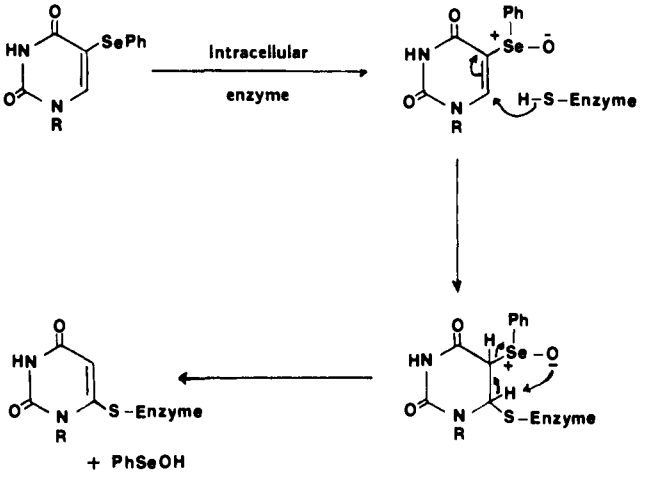

Chart I

1 PSU: X = O; R = ribosyl
2 PSC: X = NH; R = ribosyl
3 PSDU: X = O; R = 2-deoxyribosyl
4 PSAU: X = O; R = arabinosyl
5 PSAC: X = NH; R = arabinosyl

6 PSAZ

Secondly, 5-selenopyrimidines and their corresponding nucleoside or nucleotide derivatives could serve as versatile

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‡ Department of Medicinal Chemistry.
§ Department of Pharmacology.

(1) Prusoff, W. H.; Ward, D. C. *Biochem. Pharmacol.* **1976**, *25*, 1233–1239.

0022-2623/86/1829-1293$01.50/0