Enantiomeric Enrichment in the Hydrolysis of Oxazolones Catalyzed by Cyclodextrins or Proteolytic Enzymes

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Abstract: Five 5(4*H*)-oxazolones bearing substituents of variable hydrophobicity and bulkiness in positions 2 and 4 have been hydrolyzed in the presence of the proteolytic enzymes chymotrypsin and subtilisin or the α- and β-cyclodextrins. The acylamino acids produced are partially deracemized. With the enzymes, an appreciable enantiomeric enrichment is obtained only when both substituents are relatively bulky; the enantioselectivities are nevertheless quite low for enzymic reactions. With chymotrypsin, an enantiomeric excess of 76% in favor of the L isomer is observed in the hydrolysis of the 2-phenyl-4-benzyloxazolone and of 32% in favor of the D for the 2-phenyl-4-(2-carboxamidoethyl)oxazolone. There is a general inversion of stereoselectivity between chymotrypsin and subtilisin. The hydrolysis in the presence of cyclodextrin is a multistep process with formation of an acylcyclodextrin intermediate concurrent with hydrolysis. The substituent in position 2 plays an important role in controlling the enantioselectivity. When this substituent is a phenyl, the L acylamino acid is the predominant product with an enantiomeric excess of the order of 60%. When there is a methyl in position 2, the direction of asymmetric induction is reversed or the optical yield is close to zero.

Introduction

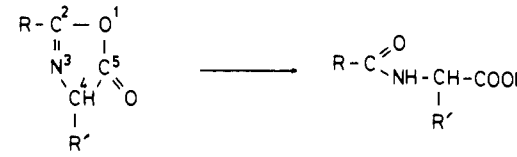
The 5(4*H*)-oxazolones have been extensively studied in the past 20 years because they are often responsible for the racemization observed in peptide synthesis.[1] On the other hand, little work has been devoted to the investigation of the possibilities of asymmetric induction during ring opening by chiral nucleophiles, the only reported example being the aminolysis of oxazolones by amino acid esters.[2] An interesting aspect of that study was that correlations were sought between the enantiomeric excesses observed in the dipeptides produced and the structure of the nucleophile or the oxazolone. In this work, we are examining the hydrolysis of oxazolones in the presence of chiral catalysts, the proteolytic enzymes chymotrypsin and subtilisin, and the α- and β-cyclodextrins and trying to find correlations between substrate structure and enantioselectivity.

The serine proteases are catalysts with complex binding and catalytic sites. They are known to catalyze the hydrolysis of a wide variety of substrates derived from amino acids or not and to do this with a stereospecificity high enough to render them useful for the resolution of substrates.[3,4] Chymotrypsin has also been shown to hydrolyze oxazolones derived from glycine, dimethylglycine, or the aromatic amino acids tyrosine and tryptophan by the usual acyl-enzyme mechanism. With the last two, the L isomer is hydrolyzed faster than the D;[5,6] the consequences of the epimerization of the substrate in the course of the hydrolysis were nevertheless not envisaged. On the other hand, the enzymic hydrolysis of several thiazolones—isosteric with the oxazolones—has been reported; the thiobenzoylamino acids produced are optically active, but apparently the authors did not measure the extent of deracemization with all the substrates.[7]

The α- and β-cyclodextrins are cyclic oligomers made up of glucose units (respectively 6 and 7) with a simple doughnut shape and presenting a hydrophobic cavity. They form inclusion complexes with a variety of guest molecules and by this process they can sometimes modify the course of their reaction.[8] They catalyze certain reactions, particularly transacylations, with some specificity and can to some extent differentiate between enantiomers.[9] When substrates like oxazolones (I) are hydrolyzed in the presence of cyclodextrins, they are likely to react like normal esters[10] by a multistep reaction with intermediate formation of an acylcyclodextrin. Then, depending on the structure of substituents in position 2 and 4, different modes of binding in the cavity might be induced which can significantly affect the enantioselectivity.

Results

Five oxazolones have been hydrolyzed in the presence of proteolytic enzymes or cyclodextrins to give the corresponding acylamino acids.



(I) (II)

a $R = C_6H_5-$, $R' = C_6H_5-CH_2-$

b $R = CH_3-$, $R' = C_6H_5-CH_2-$

c $R = C_6H_5-$, $R' = CH_3-$

d $R = CH_3-$, $R' = CH_3-$

e $R = C_6H_5-$, $R' = NH_2COCH_2CH_2-$

The choice of the substrates was based on the following considerations. For the reactions with the cyclodextrins, the oxazolones Ia–d were chosen so that they would bear a hydrophobic substituent either on both position 2 and 4, or on only one or on none. For the enzymic reactions, the oxazolones Ia and Ib were chosen as representative of substrates derived from hydrophobic aromatic amino acids for which the enzymes used, especially chymotrypsin, have a preference. The oxazolones Ic and Ie would have their most hydrophobic substituent in position 2; the substituent in position 4 would be nonhydrophobic but of different bulkiness.

When the rates of oxazolone cleavage are measured as a function of the cyclodextrin concentration, typical saturation curves are obtained indicative of complex formation before reaction. In a preliminary kinetic study,[11] we have measured the parameters k_0, k_{CD}, and K_D, respectively the rates of cleavage of the free and complexed oxazolone and the binding constant. From these, the percentage of oxazolone cleavage occurring in the complexed form could be calculated from the formula

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Table I. Enantiomeric Excess Produced in the Course of the Hydrolysis of Oxazolones Catalyzed by α- and β-Cyclodextrins

substrate	cyclodextrin	pH[a]	enant ratio L/D
Ia	α	7.86	76/24
	α	11.0	72/28
	β	7.86	79/21
	β	11.0	80/20
Ib	α	7.86	52/48
	α	11.7	51/49
	β	7.86	33/67
	β	11.7	48/52
Ic	α	7.86	77/23
	α	10.0	80/20
	β	7.86	81/19
	β	10.0	80/20
Id	α	7.86	25/75
	β	7.86	58/42

[a] For the runs at pH 7.86, the pH of the solution is brought to 10 after the oxazolone has completely disappeared.

$$\%\ \text{cleaved in (OX} \cdot \text{CD)} = \frac{k_{CD}[CD]}{k_0 K_D + k_{CD}[CD]}$$

At the cyclodextrin concentrations used for the determinations of the enantiomeric enrichments, this percentage was 80% or higher.

The ring cleavage in the presence of cyclodextrin occurs by two concurrent pathways: direct hydrolysis or formation of an acylcyclodextrin which is subsequently hydrolyzed. The infrared spectra of the reaction products of 1 mol of oxazolone Ib or Ic per mol of α-cyclodextrin at pH 7 after solvent evaporation show peaks at 1735, 1650, and 1560 cm^{-1} indicative of the presence of an acylcyclodextrin; furthermore, after disappearance of the substrate, there remains a product giving a positive reaction with Hestrin's hydroxamic acid test.[12] The percentage of oxazolone cleavage leading to acylation can be estimated by following the reaction on the pH stat. When the oxazolones are incubated with a cyclodextrin at pH 7.8, there is first a relatively fast proton release corresponding to the direct hydrolysis and eventually in the initial stage formation of some enolate; however, the amount of NaOH consumed is significantly lower than the stoichiometric quantity. When the pH is brought to 10, the acylcyclodextrin is hydrolyzed at a measurable rate. From the respective size of the low-pH and high-pH steps, the percentage of oxazolone forming an acylcyclodextrin can be estimated. It is around 45–50% for oxazolone Id with both cyclodextrins, around 55% for the oxazolones Ia and Ic with α-cyclodextrins, and around 70% in other cases.

The enantiomeric enrichments observed in the products are collected in Table I. They have been determined at pH 7.8 in the presence of phosphate buffer or at pH 10 and higher, in which case the hydrolysis of the acylcyclodextrin intermediate is fast enough for the system to become potentially catalytic. The pH change did not affect the results with the 2-phenyloxazolones but it was very damaging for the 2-methyl-4-benzyloxazolone. The substituent in position 2 appears to play an important directing role with both cyclodextrins; when this substituent is a phenyl, the L isomer is produced with a reasonable enantiomeric excess. When there is a methyl in position 2, the direction of asymmetric induction is reversed or the optical yields are close to zero.

The results obtained with the enzymes are collected in Table II. In most experiments, the substrate is added continuously at a rate corresponding to its rate of hydrolysis. With all the substrates, for a given rate of addition, there is an optimal enzyme concentration at which the enantiomeric excess in the product is maximum (runs 1–4). Preliminary kinetic studies show that this is a consequence of the fact that at low enzyme concentration the spontaneous hydrolysis predominates over the enzymic pathway; at high enzyme concentration, on the other hand, the epimerization is not fast enough compared to the rate of hydrolysis of even the slowly reacting isomer. The oxazolone can also be added as a solid powder and left to dissolve as it reacts; the optical yield in this case is nevertheless lower. It can be improved, however, by running the reaction twice (starting the second time from an already enriched substrate). With the oxazolone Ic, only a very low optical yield is obtained, but interestingly there is a reversal of stereospecificity, presumably because the phenyl in position 2 goes into the hydrophobic pocket. With the oxazolone Ie, the bulkiness of the substituent in position 4 was increased without affecting its hydrophobicity; again the D isomer is favored with chymotrypsin and an increase in enantioselectivity is observed.

Table II. Enantiomeric Excess Produced in the Course of the Hydrolysis of Oxazolones by Chymotrypsin and Subtilisin[a]

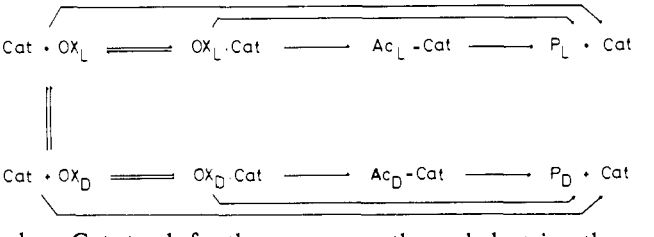
enz	OX	rate of OX addn, mol/L·s	enz concn, mol/L	enant ratio[b] L/D
CT	Ia	2.2×10^{-7}	9.00×10^{-7}	68/32
		2.2×10^{-7}	1.76×10^{-6}	88/12
		2.2×10^{-7}	2.40×10^{-6}	75/25
		2.2×10^{-7}	1.10×10^{-5}	60/40
		solid	1.90×10^{-6}	73/27
		enr solid[c]	3.68×10^{-6}	80/20
	Ib	1.2×10^{-6}	1.25×10^{-5}	65/35[d]
	Ic	2.9×10^{-6}	2.14×10^{-5}	48/52
	Ie	5.9×10^{-7}	1.00×10^{-6}	34/66
subt	Ia	4.3×10^{-7}	1.38×10^{-6}	40/60
	Ib	1.3×10^{-6}	1.14×10^{-5}	49/51
	Ic	2.0×10^{-6}	9.90×10^{-6}	54/46
	Ie	1.1×10^{-6}	7.2×10^{-6}	72/28

[a] Obtained when running the reactions at 25 °C, pH 7.0, in the presence of 0.1 M phosphate buffer. [b] Except for the oxazolone Ia with chymotrypsin, the values reported are the best values actually obtained from between three and five experiments (at various enzyme concentrations) per enzyme substrate couple; no complete optimization has been attempted yet. [c] Starting from a partially deracemized oxazolone 70% L and 30% D. [d] In the presence of 0.5 M phosphate buffer.

With subtilisin, only modest optical yields have been obtained so far; however, a very surprising result is that there is a general reversal of stereospecificity. With the derivatives of phenylalanine, the D isomer is produced in excess; consistent with this logic, with the oxazolone Ie, the L isomer is the predominant product.

Discussion

A common kinetic scheme can be written to describe the reactions:



where Cat stands for the enzymes or the cyclodextrins; there are noncovalent complexes (OX_L·Cat and OX_D·Cat) and acylated catalysts (Ac_L–Cat and Ac_D–Cat).

Cyclodextrin-Catalyzed Hydrolyses. With the cyclodextrins, the percentage of the reaction going through the acylcyclodextrin pathway does not exceed 75%. In other words, the cyclodextrins do not protect the bound substrates against direct

hydrolysis. Yet the hydroxyl groups of the amylose compete effectively with water to attack the substrate compared to what would be observed if the individual glucose units or an alcohol of similar pK_a would be present in the medium at a concentration around 10^{-2} M.

A few reactions involving covalent intermediates between the cyclodextrins and stereochemically stable optically active substrates have been reported. Kitaura and Bender obtained a k_L/k_D ratio of 2.0 in the acylation of cycloheptaamylose by *N*-acetyl-L-phenylalanine *m*-nitrophenyl esters.[13] A larger enantioselectivity, with a reactivity ratio of 6.9, was observed in the α-cyclodextrin-catalyzed cleavage of 3-carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy *m*-nitrophenyl ester; with this substrate, however, there was no asymmetric specificity with the β-cyclodextrin.[14] The best results reported have been obtained with chiral organophosphates where the ratio of second-order rate constants (k_2/K_D) is higher than 210 in one instance.[15] With these substrates, however, the center of chirality is the phosphorus atom that is being attacked; this may explain the very high enantioselectivity.

The enantiomeric enrichments reported here reflect overall reactivity ratios k_L/k_D; these amount to about 4 in the best cases. When appreciating this value, it should be realized that the enantiomeric excess observed in the final product is the result of the interplay of several factors. First, if the binding constants of the enantiomers are not equal, the epimerization reaction will tend to shift the equilibrium in favor of the oxazolone giving the most stable complex. Second, several competing pathways lead from the oxazolone to the final product: hydrolysis in solution, hydrolysis in the complex, acylation of the cyclodextrin. The k_L/k_D ratio must be zero for the first pathway and is probably different for the last two; one might speculate that the acylation pathway will have a larger ratio because in this case the substrate has to come in closer contact with the chiral centers of the host. Finally, under the conditions of the experiments, the rate of epimerization may not be fast enough to allow the maximum enantioselectivity to be reached. A detailed kinetic study is being conducted to shed some light on these questions.

Whatever the details of the mechanism, one observation made in this study is worth emphasizing. The enantiomeric ratio is largely dependent on the structure of the substituent in position 2 and there is a reversal or a significant drop in the enantioselectivity when the substituent is changed from phenyl to methyl. This result should be contrasted with the data on the aminolysis of oxazolones by the amino acid esters.[2] In this case, the enantiomeric excess obtained depends on the structure of the substituent in position 2, but in no instance was a reversal of the k_L/k_D ratio observed, presumably because the gross relative orientation of the substrate and the nucleophile remains constant. With the cyclodextrins, the structure of the substituent in position 2 can influence the overall orientation of the substrate with regard to the nucleophilic groups of the amylose. More specifically, a phenyl in position 2 is probably able to induce a common mode of binding for the oxazolones Ia and Ic. Eventually, if there are two modes of binding for the oxazolone Ia, the complex with the benzyl nucleus in the cavity is transformed less rapidly and consequently contributes little to the enantioselectivity.

Enzymic Hydrolyses. When acting on a specific substrate, the proteolytic enzymes chymotrypsin and subtilisin catalyze hydrolysis reactions very efficiently. To be recognized as specific, a substrate must be derived from an amino acid, the amino group must be protected as an amide so that the NH can form a hydrogen bond with a carbonyl group on the protein, the side chain must be hydrophobic and preferentially aromatic, and the α carbon must bear a hydrogen atom.[3] When these features are present, the enantioselectivity in favor of the L isomer is high.[16,17] On the other hand, it drops if one of them is missing and eventually a reversal can be observed as, for instance, with ethyl α-benzoyloxypropionate, $C_6H_5COO-CH(CH_3)COOC_2H_5$, where the side chain of the hydroxy acid is much less hydrophobic than the protecting group; then the ratio of k_{cat}/K_m is 18 in favor of the D isomer.[18] Nevertheless, the enantioselectivity remains large enough to render these enzymes, especially chymotrypsin, useful for resolution applications for a large variety of ester substrates not derived from amino acids.

As judged from the relatively low to very low enantioselectivities observed here, the oxazolones, even those derived from aromatic amino acids, appear as nonspecific substrates. An appreciable selectivity can be obtained only when the substituents in position 2 and 4 are bulky enough so that the substrate can take advantage of the global chirality of the active site. Moreover, if the reversal of enantioselectivity observed with the α-benzoyloxypropionate esters is retained in the 2-phenyl-4-methyloxazolone, it has dropped to nearly zero. Clearly, the absence of the NH group and the tying together of the amino-protecting group and the leaving group have a very deleterious effect on the enantioselectivity. It would be interesting to know whether this is a general rule applicable to systems in which the function to be hydrolyzed is part of a cyclic structure. Unfortunately, there is very little data on that in the literature; the only example that we are aware of is the hydrolysis of the mevalonic lactone, where no stereoselectivity is observed.[19]

Subtilisin is a bacterial protease generally considered to be very similar to chymotrypsin not only in mechanism but also in the gross stereochemistry of the active site,[20] although differences in specificity have been detected.[4,21] Consequently, the general reversal of enantioselectivity between chymotrypsin and subtilisin is a very surprising observation and to our knowledge has no precedent. When the normal recognition elements are lost by formation of the azolactone structure, factors like the overall shape of the active site and the relative hydrophobicity of the primary binding site, the acyl, and leaving group sites become dominant. These are known to be different between the two enzymes.

Conclusions

Although there are similarities between the cyclodextrins and the proteolytic enzymes, one major difference as far as asymmetric induction is concerned is that with the cyclodextrins it is not necessary to have large substituents both in position 2 and 4 to obtain a significant enantiomeric excess. This observation can be interpreted if it is realized that the global chirality of the cyclodextrin binding site is poor; on the other hand, the secondary hydroxyl groups that are acylated in the course of the reaction are attached to asymmetric carbons.

The enantiomeric excess obtained in the cyclodextrin-catalyzed 2-phenyloxazolones hydrolyses is hardly high enough to render the process useful for deracemization applications. Nevertheless, the directing effect of an easily removable group is interesting because it can ensure a better control of the asymmetric reaction than attainable with normal nucleophiles. One might hope that this effect will be retained in cyclodextrin molecules suitably modified to increase the enantioselectivity.

Experimental Section

Materials. α-Cyclodextrin (research grade) was bought from Serva; β-cyclodextrin hydrate (mp 298–300 °C) was bought from Aldrich; they were used without further purification.

The enzymes chymotrypsin from bovine pancreas (lyophilized, salt free), subtilisin (subtilopeptidase A) from *Bacillus subtilis* (lyophilized), aminoacylase from hog kidney (suspension in 3.2 M ammonium sulfate, 25 u/mg with *N*-acetylmethionine as substrate at 25 °C), catalase from beef liver (suspension in water, 65 000 u/mg at 25 °C),

and D-amino acid oxidase from hog kidney (suspension in 3.2 M ammonium sulfate, 15 u/mg with D-alanine as substrate at 25 °C) were bought from Boehringer.

The benzoyl- and acetyl-L- or -DL-amino acids were prepared as described in ref 22. They were recrystallized from ethanol–water: benzoyl-DL-phenylalanine, mp 165–166 °C (lit. 165–166 °C[22]); benzoyl-DL-alanine, mp 164–165 °C (lit. 165–166 °C[22]); benzoyl-L-phenylalanine, mp 145–146 °C, $[\alpha]^{25}_D$ +16.9° in 1 N KOH (lit. mp 145–146 °C, $[\alpha]^{20}_D$ −17.1° for benzoyl-D-phenylalanine[22]); benzoyl-L-alanine, mp 150–151 °C, $[\alpha]^{25}_D$ +35° in 1 N KOH (lit. mp 150–151 °C, $[\alpha]^{20}_D$ +37.1° [22]); benzoyl-L-glutamine, mp 156–157 °C; acetyl-L-phenylalanine, mp 170–171 °C (lit. 171 °C[22]).

The 2-phenyl-4-benzyl- and the 2-phenyl-4-methyloxazolones were prepared according to de Jersey et al.[23] and purified by sublimation (Ia) or vacuum distillation (Ic). Ia: mp 70–71 °C (lit. 70.5–71 °C[24]). Ic: bp 80 °C (0.5 mm), mp 38–39 °C (lit. 39 °C[25]).

2-Phenyl-4-(2-carboxamidoethyl)oxazolone was prepared according to Chen et al.:[26] mp 87–88 °C; IR in KBr 1817 and 1810 (C=O), 1658 cm^{-1} (C=N); NMR 7.92 and 7.52 (2 multiplets, phenyl), 5.5 (multiplet, NH_2), 4.50 (triplet) J = 6.5 Hz, CH), 2.72–2.30 ppm (multiplet, 2 CH_2).

The 2-methyloxazolones were prepared according to Bergmann et al.[27] Ib: bp 118 °C (1 mm); colorless oil; IR 1820 (C=O), 1660 cm^{-1} (C=N). Id: bp 91 °C (1 mm); colorless oil; IR 1825 (C=O), 1655 cm^{-1} (C=N); NMR 1.47 (doublet, J = 7 Hz, CH_3 in position 4), 1.82 (singlet, CH_3 in position 2), 4.53 (quadruplet, CH). Because of its instability, this oxazolone was prepared immediately before use and directly transferred to dry acetonitrile as a stock solution.

Methods. A. Enzymic Hydrolyses. The oxazolones are dissolved as stock solutions in acetonitrile (10^{-1} M) and added continuously to a solution containing the enzyme in the presence of phosphate buffer 0.1 M at pH 7 at a rate corresponding to the overall rate of hydrolysis. After completion of the reaction, the enzyme is removed by ultrafiltration with a membrane UM2 (Amicon, >98% retention for mol wt 25 000). The phosphate buffer is precipitated with $CaCl_2$ and filtered, the solvent is evaporated, and the product is taken up in acidic ethanol; the enantiomeric excess is determined by reading the ratio between the optical rotation at 365 nm and the absorbance at the maximum of the UV spectrum. The identity of the product is confirmed by recording the IR spectrum of the solid (KBr disk). Blank experiments were conducted to check that leakage of the enzymes or their degradation products did not contribute to the optical rotation. The values of the $[\alpha]^{25}_{365}$ determined in acidic ethanol for the reference acylamino acids are the following: acetyl-L-phenylalanine, +200°; benzoyl-L-phenylalanine, −59°; benzoyl-L-alanine, +39°; benzoyl-L-glutamine, −29°. They were measured on a Perkin-Elmer 241 polarimeter. The enzymes were titrated with cinnamoylimidazole.[28]

B. Cyclodextrin-Catalyzed Hydrolyses. The oxazolones dissolved in acetonitrile (10^{-1} M) are added continuously to solutions of cyclodextrins (10^{-2} M) at pH 7.8, 10, or 11 at a rate corresponding to the rate of reaction (to avoid precipitation of the less soluble substrates). The total oxazolone added is kept around one-fifth of the cyclodextrin concentration. When the reaction is run at pH 7.8, the solution is brought to pH 10 to hydrolyze the acylcyclodextrin formed. The identity of the final reaction product, an acylamino acid, was checked by comparing its infrared spectrum after workup with that of an authentic sample. Because of the difficulty of removing the last traces of cyclodextrin from the product, the enantiomeric enrichments were not determined by measurement of the optical rotation.[29]

For acetylamino acids, the proportion of the L isomer was determined by incubating the reaction mixture with aminoacylase previously dialyzed (thrice) against phosphate buffer to remove most of the ammonia in the enzyme preparation. The amount of ninhydrin-positive material[30] was measured immediately after enzyme addition and at several time intervals until a plateau was reached (up to 24 h). The difference between the reading at zero time and the plateau is proportional to the amount of L amino acid liberated by the enzyme. The proportionality constant is obtained from a calibration curve with the free amino acid. The validity of the method is checked by analyzing reaction mixtures of known composition: *N*-acetyl-L-phenylalanine in the presence or in the absence of the cyclodextrins released 98–99% of the theoretical amount of L-phenylalanine; *N*-acetyl-DL-phenylalanine incubated with the α-cyclodextrin gave 49% of the theoretical amount; the hydrolysis product of the 2-methyl-4-benzyloxazolone gave 49%.

For the benzoylamino acids, the method is not applicable because they are very poor substrates for the aminoacylase. At the end of the run, the reaction mixture was acidified to pH 2. The solvent was evaporated in the cold by lyophilization. The benzoylamino acid is extracted from the residue with ethanol, a solvent in which the cyclodextrins are only very slightly soluble. After solvent evaporation, the benzoyl group is hydrolytically removed by boiling for 7 h with 6 N HCl; under these conditions, the amino acid has been shown to be configurationally stable.[31] After evaporation of the HCl and extraction of the benzoic acid with ether, the amino acid is taken up in water and the pH adjusted to 7. The amount of amino acid in the sample is determined with the ninhydrin reagent. In this method, some of the amino acid is lost, presumably during the extraction step. Typical recoveries were for benzoylphenylalanine with α-CD, between 70 and 74%; with β-CD, between 87 and 89%; for benzoylalanine with α-CD, between 70 and 80%; with β-CD, between 72 and 78%. The percentage of D-amino acid in the sample was measured by stereospecific oxidation with a D amino acid oxidase according to Walsh et al.[32] Sample (2 mL) is incubated for 3 h at 30 °C with 20 μL of D amino acid oxidase in the presence of catalase to prevent the oxidative decarboxylation of the α-keto acid formed. The α-keto acid is then measured by incubating 0.25 mL of the sample with 2.5 mL of 0.1 M solution of thiosemicarbazide at pH 5.4 against a blank identical in composition except that the enzymes were omitted.

The amount of L amino acid remaining after oxidation is checked in two instances: the solution was first brought to pH 13 and evaporated to dryness twice to remove the ammonia, the residue then taken up in a known volume of buffer, and the pH adjusted to 7. The amino acid content is determined with ninhydrin.

Because some of the amino acid is lost in the method just described, it was felt necessary to check that the enantiomeric enrichment was not an artifact of the extraction step. In several runs, the cyclodextrin benzoylamino acid mixture was submitted directly to acid hydrolysis and the analysis continued as described above. In this variant, the cyclodextrin is extensively degraded giving light brown solutions. The measurements are done against a blank of cyclodextrin similarly treated. In this method, 96 ± 1% of the amino acid is recovered. The two method gave consistent results within 1 or 2%.

In blank experiments, known mixtures of benzoyl-DL-phenylalanine and α-cyclodextrin were analyzed. With an extraction step, 82% of the total amino acid was recovered of which 47% was oxidized with the D amino acid oxidase; without an extraction step, the corresponding percentages are 93 and 48%.

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23672 RP, a New Macrolide Antibiotic from *Streptomyces chryseus*. Mass Spectrometry Study and X-ray Structure Determination

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Abstract: A new macrolide antibiotic, 23672 RP, has been isolated from *Streptomyces chryseus* DS 12370 (NRRL 3892). It has some activity in vitro and in vivo against mycobacteria. Structural studies have been run by mass spectrometry and X-ray crystallography, the complete structure being established by the latter technique. 23672 RP has a 14-membered lactonic ring resembling that of lankamycin. Three sugar units are bonded to C-3, C-5, and C-15, and an α-hydroxyisovalerate chain is linked to C-11.

In the course of the research of new antimicrobial agents, 23672 RP was isolated from the culture broth of *Streptomyces chryseus* DS 12370 (NRRL 3892). This compound is a neutral macrolide according to its physical and chemical properties. It is atoxic in the mouse at the dose of 2.5 g/kg when administered by subcutaneous route, and it has some activity in vitro and in vivo against Gram-positive bacteria and mycobacteria without any detectable activity against Gram-negative bacteria. The following minimum inhibitor concentrations were determined (μg/mL): *Staphylococcus aureus* ATCC 6538P, 0.54; *Sarcina lutea* ATCC 9431, 0.07; *Bacillus cereus* ATCC 6630, 0.10; *Mycobacterium* species ATCC 607, 0.42; *Mycobacterium* H37Rv, 1.25; *Escherichia coli* ATCC 9637, >200. The compound is effective in protecting mice from lethal infections produced by staphylococci; the curative doses (CD_{50} values) in mice are 35 and 85 mg/kg by subcutaneous and per os routes, respectively. Against a mycobacterial infection by virulent human strain, the compound at the doses of 100–200 mg/kg by subcutaneous or per os routes increases significantly the life of treated animals comparatively to the reference.[2] We wish to report here studies on this compound by mass spectrometry and X-ray crystallography, the structure being established by the latter technique.

Experimental Section

A. Mass Spectrometry. 23672 RP has been studied by field desorption, chemical ionization, and electron impact mass spectrometry.

Field Desorption. Spectra have been carried out on a Varian MAT 311A apparatus by the cationization technique upon addition of potassium iodide. The result is given in Figure 1 with the peak at M + K 1017: M = 978. The three major fragments are *m/e* 129, 145, and 175.

Chemical Ionization. Spectra have been recorded on a Finnigan 4000 processed by the Data System 6100, using isobutane as reactant gas. The chemical ionization mass spectrum of 23672 RP is reproduced in Figure 2. The protonated molecular ion (MH^+) gives the peak at *m/e* 979; the prominent peaks in the low-mass portion of the spectrum correspond to fragment ions of the sugar residues at *m/e* 129, 145, and 175.

Electron Impact. The spectrum has been recorded on Finnigan 3300 and AEI MS50 apparatus, this last one processed by the Data System DS 50. Not unexpectedly, 23672 RP does not display a detectable molecular ion; the peak of highest mass occurs at *m/e* 689. Some of the fragments have been measured exactly in order to establish the formula at *m/e* 129, 145, and 175. Three sugars have been displayed in the form of oxonium ions: 129.0550, oxonium ion $C_6H_9O_3$, sugar $C_6H_{10}O_4$; 145.0875, oxonium ion $C_7H_{13}O_3$, sugar $C_7H_{14}O_4$; 175.0998, oxonium ion $C_8H_{15}O_4$, sugar $C_8H_{16}O_5$. The two last sugars may correspond to mycarose and mycinose.

B. X-ray Diffraction. Experimental. The sample of 23672 RP was crystallized from an aqueous acetone solution. Crystals belong to the monoclinic space group $P2_1$; the unit cell dimensions are a = 11.147 (3) Å, b = 18.350 (4) Å, c = 14.564 (4) Å, β = 93.59 (5)°, Z = 2.

A crystal with approximate dimensions 0.33 × 0.38 × 0.41 mm was mounted on a Philips PW1100 four-circle diffractometer. Intensity data were collected by the use of Ni-filtered Cu radiation, up to 2θ = 136°. Background was estimated from measurements outside reflections between θ = 2° and θ = 66°; 3888 integrated intensities out of 5530 independent reflections were above $3\sigma(I)$, where $\sigma(I)$ is the standard deviation derived from counting statistics. No absorption correction was applied.

Structure Determination and Refinement. The structure was solved by direct methods. The starting set was formed with three reflections fixing the origin, and six symbols which were chosen in Σ_2[3a] and positive quartets[3b] listings as reflections having the more numerous interactions. This set has been tested with Riche's phase function,[4] but too many solutions appeared. The four well-defined symbols were selected in a multisolution program,[5] in which the phases were generated by the magic integers procedure,[6] and where the solutions are ranked according to the NQ test[3b] and residual.[7] This procedure was not sufficient to provide the solution. We then added two Σ_1 relations[3a] in the starting set. This time, the set with the minimal R and the more negative NQ value led to the solution.

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