ENZYMATIC DIMERIZATION OF PENICILLIN X

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(Received for publication August 3, 1992)

Penicillin X methyl ester was transformed into three types of dimer by laccase from *Coriolus versicolor*. The dimers are considered to be formed by free-radical addition of phenoxy radicals produced by laccase. The enzyme reaction with the ester as substrate was more suitable for forming dimers than that with the sodium salt as substrate. Penicillin X pivaloyloxymethyl ester was also transformed into a dimer, which had antibacterial activity in the presence of esterase.

Enzymatic and microbial transformations are convenient methods to synthesize derivatives of various classes of antibiotics. The advantages of enzyme-catalyzed reactions are that they take place under mild reaction conditions and provide stereoselectivity and specificity of the reaction. Many enzyme-catalyzed reactions, such as oxidation, reduction, alkylation, dealkylation, acylation[1], deacylation[2], hydrolysis, phosphorylation[3], and glycosylation[4], have been used for transformations of antibiotics. However, phenolic oxidative coupling reaction has not yet been applied to enzymatic transformation of antibiotics. The reaction is considered to be applicable for the synthesis of a new antibiotic by combining two antibiotics containing a phenolic moiety or by introducing a phenolic compound into an antibiotic containing a phenolic moiety.

For this purpose we employed laccase as a polyphenol oxidase. Laccase is a copper-containing enzyme and generally catalyzes the removal of hydrogen from phenolic hydroxyl groups using molecular oxygen as a primary electron acceptor to give phenoxy radicals, which undergo free-radical addition to form oligomeric products[5~9]. As the first attempt, we applied the reaction to form dimers of penicillin X (PcX), because PcX has a phenolic side chain and a PcX dimer might be supposed to have antibacterial activity. Our original attempt to dimerize PcX sodium salt by laccase was, however, unsuccessful because of the instability of the reaction products. Therefore, PcX esters were used as substrates and the compounds were transformed into dimers.

In the present paper, we describe the formation of three types of PcX methyl ester dimers, the subsequent structure elucidation, and the application to PcX pivaloyloxymethyl ester.

Materials and Methods

Laccase

Laccase used in this study was purified from culture solutions of the fungus *Coriolus versicolor* IFO

9791 according to the described method[10]. One unit of laccase is defined as the amount of enzyme oxidizing 1 μmol of syringaldazine per minute in 50 mM sodium phosphate buffer (pH 6.0) at 30°C[11].

Preparations of PcX Methyl Ester and Pivaloyloxymethyl Ester

PcX was prepared by acylation of 6-aminopenicillanic acid with 4-hydroxyphenylacetic acid through the corresponding active ester. The compound was then transformed into the methyl ester (PcXOMe) and the pivaloyloxymethyl ester (PcXPOM) by reacting the sodium salt with methyl iodide and pivaloyloxymethyl chloride, respectively, in DMF.

Dimerization of PcXOMe

PcXOMe was dimerized using two-phase systems composed of an organic solution of PcXOMe and a buffer containing laccase: Two hundred and thirty mg of PcXOMe was dissolved in 23 ml of chloroform in a 500-ml Erlenmeyer flask, and then 20 ml of 0.1 M sodium phosphate buffer (pH 6.0) containing 11×10^3 units of laccase was added into the flask. The flask was capped and incubated at 28°C for 18 hours on a rotary shaker.

Dimerization of PcXPOM

PcXPOM was allowed to react in suspension: One hundred mg of PcXPOM was dissolved in 10 ml of chloroform. The solution was poured into a 2-liter Erlenmeyer flask, and then the solvent was removed under reduced pressure to coat the bottom of the flask with PcXPOM. One hundred ml of 10 mM sodium phosphate buffer (pH 6.0) containing 11×10^3 units of laccase was added into the flask. The flask was slowly shaken on a reciprocal shaker at 30°C for 20 hours.

HPLC Analysis

Progress of the reaction was monitored by HPLC on a YMC-Pack s-5 60A SIL (4.6 × 250 mm) column using a 2-propanol concentration gradient in chloroform from 0% to 10% for 60 minutes with a flow rate of 1.0 ml/minute. Products and substrate, PcXOMe or PcXPOM, were detected by measuring the absorbance at 280 nm. In the reaction of PcXOMe the chloroform layer was used for HPLC analysis. In the reaction of PcXPOM the reaction mixture was extracted with an equal volume of EtOAc, and the extract was used for HPLC analysis.

Fig. 1. HPLC analysis of the reaction mixture of PcXOMe.

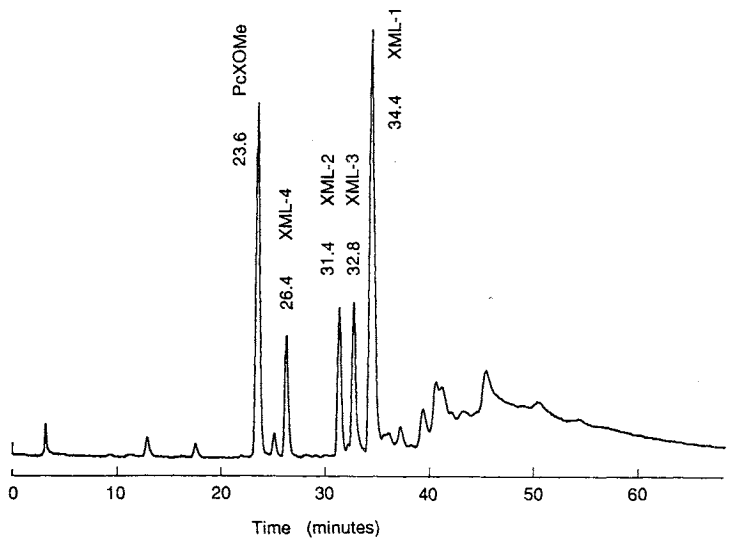
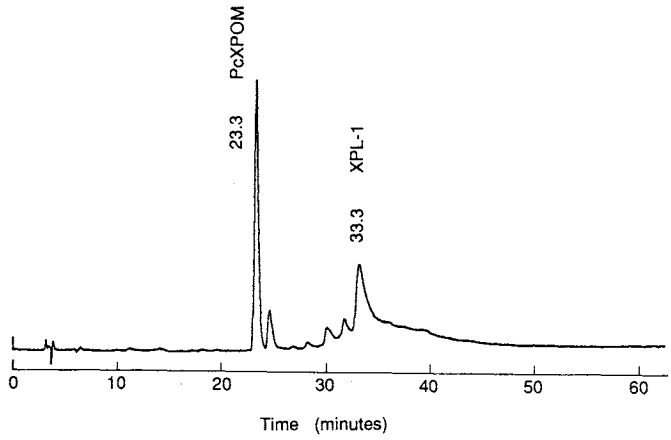


Fig. 2. HPLC analysis of the reaction mixture of PcXPOM.



Isolation and Purification of PcXOMe Dimers

The products existed in the organic layer of the two-phase systems, and four main products were named XML-1, 2, 3, and 4, whose retention times were 34.4, 31.4, 32.8, and 26.4 minutes, respectively (Fig. 1). After the reaction the organic solution was separated, dried over anhydrous sodium sulfate, and concentrated under reduced pressure to a small volume. The concentrate was applied on a preparative silica gel column of YMC-Pack s-10 120A SIL (2 × 25 cm) and developed with a mixture of chloroform and 2-propanol (50 : 1) at a flow rate of 7.0 ml/minute. Elution was monitored by UV adsorption at 280 nm. The peak fractions of these four products were collected separately and then concentrated to dryness under reduced pressure to give 18 mg of XML-1, 8 mg of XML-2, 12 mg of XML-3, and 8 mg of XML-4 as pure compounds.

Isolation and Purification of a PcXPOM Dimer

From HPLC analysis of the reaction mixture, a main peak at the retention time of 33.3 minutes was named XPL-1 (Fig. 2). The reaction solution was extracted twice with an equal volume of EtOAc. The combined extract was dried over anhydrous sodium sulfate and concentrated under reduced pressure to dryness. The residue was dissolved in a small volume of chloroform and subjected to preparative HPLC, whose conditions were the same as above except for a mobile phase (chloroform - 2-propanol, 100 : 3). The eluate containing XPL-1 was concentrated to dryness under reduced pressure to give 14 mg of XPL-1 as a pure compound.

Results

Laccase-catalyzed Dimerization of PcXOMe

PcXOMe was transformed into dimers, XML-1, 2, 3, and 4, by laccase-catalyzed oxidative coupling reaction. The reaction was stopped by separating the two phases when the formation rate of XML-1 reached its maximum. The laccase used for the reaction still retained more than 70% of the activity and could be used again. PcXOMe was also transformed in suspension to produce four peaks at retention times corresponding to XML-1, 2, 3, and 4 by the similar method for dimerizing PcXPOM as described in the Materials and Methods. However, since PcXOMe was more stable in organic solution than in aqueous solution, the two-phase systems were used.

Structure Determination of XML-1, 2, 3, and 4

The structures of XML-1, 2, 3, and 4 were determined by their IR, MS and NMR spectra analyses.

Table 1. ^{1}H NMR data of XML-1, 2, 3, and 4.

Proton	XML-1	XML-2	XML-3	XML-4
2,2a-$(CH_3)_2$	1.43 s (6H), 1.48 s (6H)	1.45 s (3H), 1.46 s (3H), 1.50 s (3H), 1.58 s (3H)	1.46 s (3H), 1.49 s (3H), 1.50 s (3H), 1.62 s (3H)	1.46 s (3H), 1.47 s (3H), 1.51 s (3H), 1.54 s (3H)
3,3a-H	4.40 s (2H)	4.37 s, 4.43 s	4.39 s, 4.48 s	4.40 s, 4.41 s
$COOCH_3$	3.76 s (6H)	3.77 s (3H), 3.79 s (3H)	3.77 s (3H), 3.79 s (3H)	3.77 s (6H)
5,5a-H	5.51 d (4.1) (2H)	5.53 d (4.1), 5.54 d (4.1)	5.53 d (4.1), 5.54 d (4.1)	5.50 d (4.4), 5.53 d (4.4)
6,6a-H	5.62 dd (4.1, 8.8) (2H)	5.63 dd (4.1, 8.8), 5.69 dd (4.1, 9.3)	5.67 dd (4.1, 9.1), 5.68 dd (4.1, 8.8)	5.64 dd (4.4, 8.8), 5.65 dd (4.4, 8.8)
6,6a-NH	6.57 d (8.8) (2H)	6.15 d (9.3), 6.52 d (8.8)	6.41 d (9.1), 6.49 d (8.8)	6.09 d (8.8), 6.13 d (8.8)
4′-CH_2	3.54 d (15.9), 3.59 d (15.9)	2.82 d (14.8), 3.00 d (14.8)	2.81 d (14.3), 2.91 d (14.3)	3.49 s (2H)
10′-CH_2	3.54 d (15.9), 3.59 d (15.9)	3.58 s (2H)	3.56 d (15.7), 3.60 d (15.7)	3.61 s (2H)
1′-OH	7.52 br s	—	—	n.i.
2′-H_2	—	3.00 dd (17.3, 2.4), 3.14 dd (17.3, 4.1)	3.00 dd (17.5, 3.3), 3.07 dd (17.5, 4.1)	—
3′-H	7.15 d (2.2)	5.09 m	5.04 m	6.78 d (2.2)
5′-H	7.11 dd (2.2, 8.2)	6.54 dd (10.2, 1.6)	6.63 dd (10.4, 1.9)	6.95 dd (2.2, 8.1)
6′-H	6.87 d (8.2)	5.98 d (10.2)	5.99 d (10.4)	7.04 d (8.1)
7′-OH	7.52 br s	—	—	—
8′-H	—	—	—	7.02 d (8.1)
9′-H	7.15 d (2.2)	7.17 d (1.9)	7.21 d (1.9)	7.26 d (8.1)
11′-H	7.11 dd (2.2, 8.2)	7.08 dd (1.9, 8.2)	7.12 dd (1.9, 8.2)	7.26 d (8.1)
12′-H	6.87 d (8.2)	6.80 d (8.2)	6.79 d (8.2)	7.02 d (8.1)

Spectra were measured in $CDCl_3$ at 400 MHz. Chemical shifts are expressed by δ (ppm) (J=Hz) from internal TMS.
n.i.: Not identified.

All IR spectra of these compounds indicated a band at 1775~1780 cm^{-1} assigned to the β-lactam carbonyl group. The molecular weights of these compounds were established as 726 from the SI-MS spectra which gave molecular ion peaks at m/z 727 $(M+H)^+$. The result suggested that these compounds were dimers of PcXOMe (the molecular weight is 364). The ^{1}H and ^{13}C NMR data of these compounds are shown in Tables 1 and 2, respectively. The data were compared with those of PcXOMe (data not shown). The protons in the penam nucleuses of these compounds were virtually identical in terms of their chemical shifts and coupling constants, indicating that the penam nucleuses were inert in the coupling reaction. The conclusion was also supported by the ^{13}C NMR data.

Chemical structures of XML-1, 2, 3, and 4 were determined as shown in Fig. 3. The number of ^{13}C signals of XML-1 was seventeen that was two more than those of PcXOMe. Therefore, XML-1 was deduced to be an intramolecular symmetric compound. The two doublets (each 2H, J=8.5 Hz) at δ_H 6.81 and 7.12 in PcXOMe changed into a doublet (J=8.2 Hz) at δ_H 6.87, a doublet of doublets (J=2.2 Hz and 8.2 Hz) at δ_H 7.11 and a doublet (J=2.2 Hz) at δ_H 7.15 in the case of XML-1. This coupling pattern indicated that XML-1 had a typical 1′,2′,4′-substituted ring system. Therefore, C–C coupling was suggested to take place at the *ortho* positions in the two phenol groups.

The ^{1}H and ^{13}C NMR data of XML-2 were virtually the same as those of XML-3, which indicated these compounds were stereoisomers. In the ^{1}H NMR spectra of XML-2 and 3, 5′-H and 6′-H were up-field shifted by 0.5~0.9 ppm in comparison with those in XML-1, indicating that the two protons were olefin

Table 2. ^{13}C NMR data of XML-1, 2, 3, and 4.

Carbon	XML-1	XML-2	XML-3	XML-4
2,2a	64.8	64.7, 65.2	64.8, 65.2	64.7
2,2a-(CH_3)$_2$	26.8, 31.7	26.9, 27.1, 31.3, 31.7	26.9, 27.1, 31.6, 31.8	26.9, 27.0, 31.7, 31.8
3,3a	70.4	70.3, 70.6	70.4, 70.5	70.4
5,5a	68.0	67.7, 68.0	67.6, 68.0	68.0
6,6a	58.8	58.5, 58.6	58.5, 58.8	58.7
7,7a	171.2	167.9, 170.5	168.0, 170.6	170.1, 170.2
3,3a-CO	173.6	173.2, 173.7	173.2, 173.7	173.5
$COOCH_3$	52.4	52.3, 52.5	52.5	52.5
$CONH$	168.1	168.0	167.9	168.1
1′	152.6	195.0	195.0	155.9
2′	126.6	38.6	38.4	146.9[c]
3′	132.6	85.9	85.8	125.9
4′	125.6	47.0	47.0	129.5[d]
4′-CH_2	42.3	42.6[a]	42.6[b]	42.6[e]
5′	130.5	146.1	146.2	130.6
6′	117.7	124.1	124.2	116.9
7′	152.6	157.9	157.9	143.7[c]
8′	126.6	131.5	131.0	118.7[f]
9′	132.6	131.1	131.0	131.2
10′	125.6	127.2	127.5	126.3[d]
10′-CH_2	42.3	43.1[a]	43.1[b]	42.5[e]
11′	130.5	127.8	127.5	131.2
12′	117.7	111.1	111.1	119.8[f]

Spectra were measured in $CDCl_3$ at 100 MHz. Chemical shifts are expressed by δ (ppm) from internal TMS.
[a~f]: Assignments could be interchanged.

protons. The coupling constant between 5′-H and 6′-H ($J=10.2$ or 10.4 Hz) indicated that 5′-H and 6′-H were *cis* oriented. Furthermore, a carbon signal attributable to a ketone group was observed at δ_C 195.0 in the ^{13}C NMR spectra. Both XML-2 and 3 had a characteristic ultraviolet absorption maximum at 221 nm in methanol. These findings suggested the presence of a *cis*-α,β-unsaturated ketone moiety. The ^1H-^1H COSY experiments of XML-2 and 3 revealed methylene protons, 2′-H_2, were coupled to methine proton, 3′-H. Coupling pattern of other aromatic protons, 9′-H, 11′-H, and 12′-H, showed the presence of a 7′,8′,10′-substituted ring system. XML-2 and 3 were suggested to be formed by a *ortho-para* coupling of the two phenoxy radicals of PcXOMe and following addition of a hydroxyl group at 7′-position to the 3′-position of the dienone. Consequently, these compounds are diastereomers based on the chiral centers of C-3′ and C-4′.

The ^1H-^1H COSY experiment of XML-4 revealed that an aromatic proton, 3′-H, was coupled to 5′-H ($J=2.2$ Hz), and 5′-H to 6′-H ($J=8.1$ Hz), while 8′-H and 12′-H were coupled to 9′-H ($J=8.1$ Hz) and 11′-H ($J=8.1$ Hz), respectively. The coupling patterns of the two groups of aromatic protons indicated that the former group constituted a 1′,2′,4′-substituted ring system, and the later group constituted a 7′,10′-substituted ring system. XML-4 was a dimer produced by a C–O coupling of the two phenoxy radicals of PcXOMe.

Application for Dimerization of PcXPOM

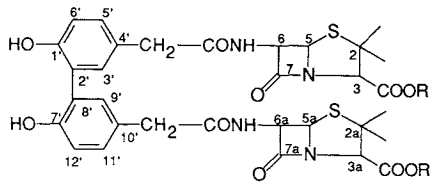
XML-1, 2, 3, and 4 were almost inactive against tested organisms (data not shown). Next, we used PcXPOM as a substrate of laccase to show that a PcX dimer has antibacterial activity, because the pivaloyloxymethyl ester group of PcXPOM was easily hydrolyzed by esterase to produce PcX. Thus, PcXPOM

Table 3. ^{1}H and ^{13}C NMR data of XPL-1.

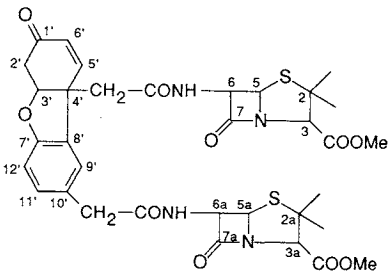
Position	δ_C	δ_H	Position	δ_C	δ_H
2,2a	64.7	—	*C*ONH	171.1	6.51 d (8.8) (2H)
2,2a-(CH_3)$_2$	26.7, 31.4	1.46 s (6H), 1.48 s (6H)	7,7a	173.6	—
3,3a	70.0	4.40 s (2H)	1′,7′	152.5	—
3,3a-*C*O	166.3	—	1′,7′-OH	—	n.i.
COO*C*H$_2$	79.9	5.75 d (5.9) (2H), 5.85 d (5.9) (2H)	2′,8′	125.5	—
			3′,9′	132.6	7.15 d (2.2) (2H)
			4′,10′	126.5	—
COOCH$_2$O*C*O	176.8	—	4′,10′-*C*H$_2$	42.2	3.55 d (16.1) (2H), 3.59 d (16.1) (2H)
COOCH$_2$OCO*C*	38.8	—			
COOCH$_2$OCOC(CH_3)$_3$	26.9	1.22 s (18H)	5′,11′	130.5	7.12 dd (2.2, 8.2) (2H)
5,5a	68.0	5.50 d (4.1) (2H)	6′,12′	117.7	6.88 d (8.8) (2H)
6,6a	58.8	5.64 dd (4.4, 8.8) (2H)			

^{1}H and ^{13}C chemical shifts were given in δ downfield of TMS at 400 and 100 MHz, respectively. Coupling constants in parentheses were given in Hz.
n.i.: Not identified.

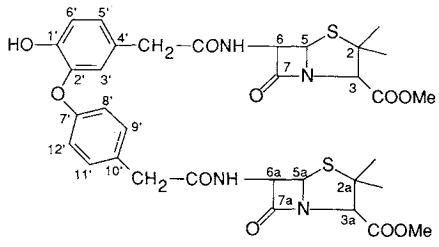
Fig. 3. Structures of XML-1, 2, 3, 4, and XPL-1.



XML-1 R = CH_3
XPL-1 R = $CH_2OCOC(CH_3)_3$



XML-2, 3



XML-4

Table 4. Antibacterial activity of XPL-1, the assay carried out after the preincubation with hog liver esterase.

Test organism	MIC (μg/ml)	
	PcX (Na)	XPL-1
Bacillus subtilis ATCC 6633	<0.006	1.56
Staphylococcus aureus 209P	0.012	0.39
S. epidermidis ATCC 12228	3.13	50
Micrococcus luteus ATCC 9341	<0.006	0.10
Escherichia coli K-12	6.25	>100

is a prodrug of PcX[12]. When PcXPOM was incubated together with laccase, a PcXPOM dimer, XPL-1, was formed. On the other hand, when PcXPOM was dissolved in chloroform or ethyl acetate and then the organic solution was mixed with phosphate buffer containing laccase as with PcXOMe, no product was formed. This is considered to be because the partition coefficient of PcXPOM in favor of the aqueous layer was so low that PcXPOM was not oxidized by laccase.

The structure determination of XPL-1 was carried out in a similar manner as those of XML compounds. The molecular weight of XPL-1 was established as 926 from positive and negative FAB-MS spectra which gave molecular ion peaks at m/z 927 $(M+H)^+$ and 925 $(M-H)^-$, respectively. This suggested that XPL-1 was a dimer of PcXPOM (the molecular weight is 464). The structure was supported by comparison the ^{1}H and ^{13}C NMR spectra of XPL-1 with those of PcXPOM and XML-1.

Finally, all the protons and carbons in XPL-1 were assigned by 2D NMR experiments including ^{1}H-detected multiple-bond heteronuclear multiple quantum coherence spectroscopy (HMBC) and ^{1}H-detected multiple quantium coherence spectroscopy (HMQC) as shown in Table 3. Chemical structure of XPL-1 was determined as shown in Fig. 3.

Antibacterial Activity of XPL-1

XPL-1 is a prodrug of a PcX dimer. The antibacterial activities of XPL-1 were evaluated by using the conventional dilution method in Mueller-Hinton broth at pH 7.0 after the preincubation with hog liver esterase (Sigma CO.) to hydrolyze the pivaloyloxymethyl ester groups. XPL-1 showed lower activity against every tested organisms than PcX (Table 4).

Discussion

We originally used PcX sodium salt as a substrate of laccase. PcX was converted to antibacterial products in part but mostly polymerized to give products without antibacterial activity. Since the yields of these antibacterial products were low and further they were unstable, we could not determine their chemical structures. The advantages of using the ester as substrate are that the ester was transformed into dimers more effectively than the sodium salt, and the produced dimers were more stable and easier to isolate than the products from the sodium salt.

When a *para*-alkylated phenol is a substrate, laccase catalyses the removal of hydrogen from the phenolic hydroxyl group of the substrate to form phenoxy radicals, in which an unpaired electron distributes over the oxygen and the positions *ortho* and *para* to it. The formations of XML-1, 2, 3, 4, and XPL-1 can be explained by free-radical addition of these three types of phenoxy radicals: XML-1 and XPL-1 were *ortho-ortho* coupling products. XML-2 and 3 were *ortho-para* coupling products. XML-4 was an *ortho-O* coupling product.

This study presents a new method to produce dimers of phenolic compounds under mild conditions. The coupling method using laccase as a catalyst is applicable to unstable compounds such as β-lactam antibiotics.

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