# FERMENTATION, ISOLATION AND CHARACTERIZATION OF ANTIBIOTIC PR-1350*

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A number of strains of *Oidiodendron truncatum* was shown to produce a new antibiotic, PR-1350, which was isolated in the form of an amorphous powder either directly or *via* a crystalline monomethanolate, PR-1381, which in solution is reconverted to the parent compound. The antibiotic inhibits a broad spectrum of Gram-positive and Gram-negative bacteria *in vitro*, and has been shown to be active against P-388 lymphocytic leukemia in mice. Biosynthetic considerations based on the results of [1-$^{13}$C]acetate incorporation indicate that the antibiotic is a diterpene of the clerodane type.

The only hitherto known antibiotic originating from the genus Oidiodendron is fuscin, which has been isolated from a strain of *Oidiodenron fuscum*[1]. In this paper we shall report the isolation of a new antibiotic, PR-1350, from a number of strains of *Oidiodendron truncatum*.

## Microorganisms

The antibiotic was first isolated from a strain of *Oidiodendron truncatum* (HL-972; CBC 475.78) isolated from a soil sample collected near Copenhagen and was subsequently shown to be produced by all of a number of strains of *O. truncatum* tested, whereas none of the examined strains of other *Oidiodendron* species produces any antibacterial activity (Table 1).

## Physico-chemical Properties

PR-1350 can be obtained as an amorphous colorless powder by precipitation with cyclohexane from an ethyl ether solution. Alternatively, a colorless crystalline material can be obtained from a solution of PR-1350 in methanol. This crystalline material is called PR-1381. The IR-spectra of PR-1350 and PR-1381 are shown in Figs. 1 and 2. The two compounds show identical mass spectra and an UV maximum was observed for both at 230 nm ($E^{1\%}_{1cm}$ 310). On the other hand, the optical rotations of freshly prepared solutions of the two compounds differ considerably. However, the optical of such solutions change in the course of some hours and, eventually, the two solutions show comparable rotations values (Table 2).

An NMR spectrum of PR-1381 can be obtained when the spectrum is run immediately after preparation of the solution, and the best resolved spectrum is that obtained in DMSO-$d_6$ (Fig. 3). However, when the solution is left at room temperature the appearance of the spectrum changes during the next few hours. The end point of a similar transformation in $CDCl_3$ solution is shown in Fig. 4 together with the NMR spectrum of PR-1350 in $CDCl_3$. The similarity of these two spectra is obvious, the

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Table 1. Production of antibiotics by a number of *Oidiodendron* species.

| Organism | Number | Zone of inhibition (mm) after 6 days (*S. aureus*) | Organism | Number | Zone of inhibition (mm) after 6 days (*S. aureus*) |
|---|---|---|---|---|---|
| *O. ambigium* | CMI 89334 | 26*,** | *O. pilicola* | CBS 141.72 | — |
| *O. chlamydosporicum* | ATCC 18448 | — | *O. rhodogenum* | CBS 237.31 | — |
| *O. cerealis* | CBS 321.31 | — | *O. tenuissimum* | CBS 238.31 | — |
| *O. citrinum* | CBS 400.69 | — | *O. truncatum* | CBS 114.65 | 29* |
| *O. echinulatum* | CBS 113.65 | — | *O. truncatum* | CBS 115.65 | 31* |
| *O. flavum* | CMI 96251 | — | *O. truncatum* | CBS 222.65 | 30* |
| *O. fuscum* | CMI 89370 | — | *O. truncatum* | CBS 629.70 | 29* |
| *O. griseum* | CBS 249.33 | — | *O. truncatum* | CMI 99669 | 26* |
| *O. kalrai* | ATCC 18434 | — | *O. truncatum* | Leo HL-972, CBC 475.78 | 36* |
| *O. maius* | CBS 402.69 | — | | | |
| *O. periconioides* | CBS 391.69 | — | | | |

* PR-1350 was isolated and identified by NMR from all of the 7 strains showing inhibition of *S. aureus*.
** Reinvestigation at Centraalbureau voor Schimmelcultures, Baarn, showed this strain to be an *Oidiodendron truncatum*.

Fig. 1. IR spectrum of PR-1350 (KBr).

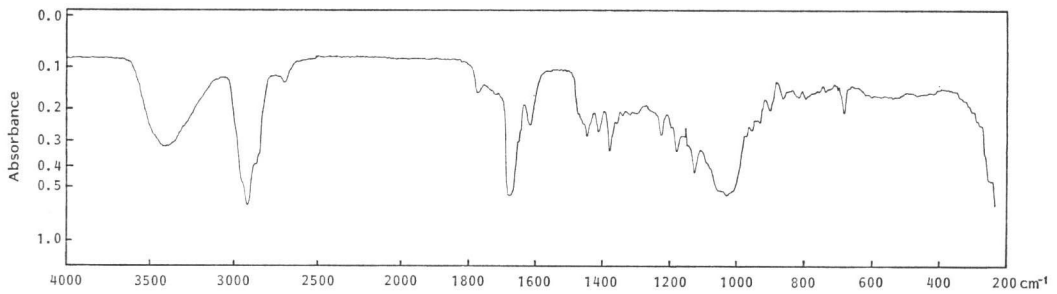
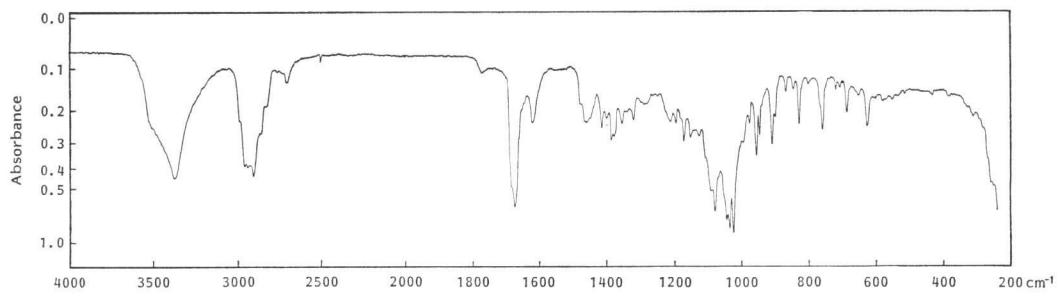



Fig. 2. IR spectrum of PR-1381 (KBr).




small differences being easily explained by the presence of one mol of methanol in the spectrum in Fig. 4A.

From these observations it is concluded that PR-1381 is formed by addition of one mol of methanol to PR-1350, and that PR-1381 in solution readily loses the methanol with regeneration of PR-1350.

The complex nature of the spectra in Fig. 4 contrasting the relative simplicity of the initial spectrum of the methanolate, shown in Fig. 3, suggests that PR-1350 in solution exists in a number of equilibrating

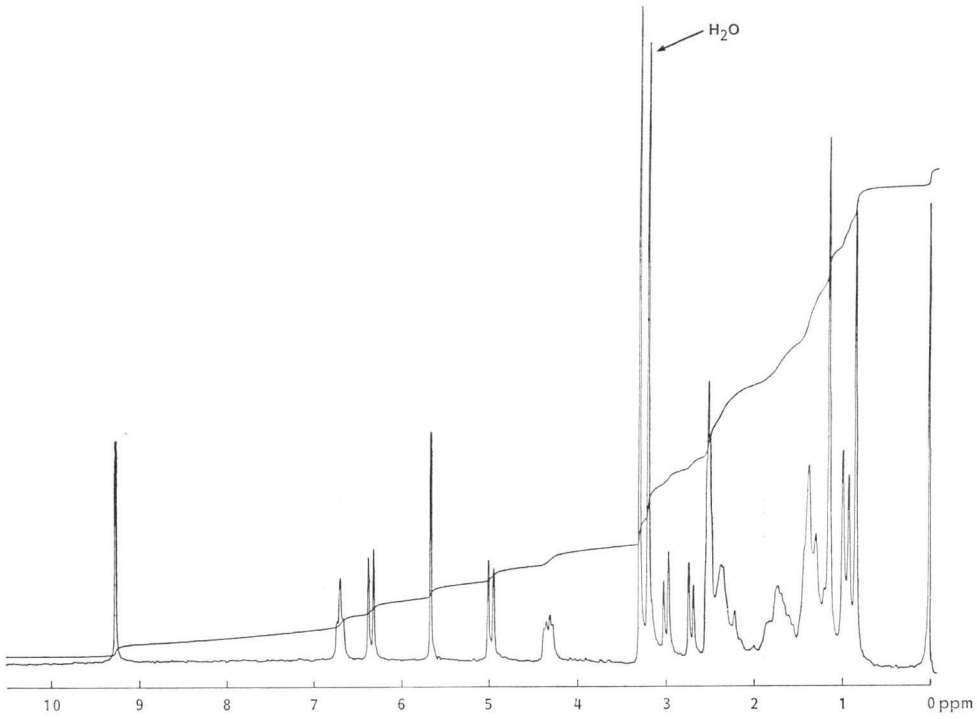
forms. In accordance with this, rapid chromatography of PR-1350 on silica gel or Sephadex LH-20 elutes the antibacterial activity in several partly separated bands. However, rechromatography of the content of one of these gives rise to the reappearance of all the bands, thus implying that the separated forms of PR-1350 are rapidly transformed to the original mixture.

Table 2. The specific rotation of PR-1350 and PR-1381.

| Time after preparation of the solution (hours) | $[\alpha]_D^{20}$ ($c$ 1, EtOH) | |
|---|---|---|
| | PR-1350 | PR-1381 |
| 0 | +82.1° | +102.8° |
| 1 | +85.6° | +94.0° |
| 2 | +86.5° | +91.6° |
| 4 | +86.6° | — |
| 10 | — | +89.6° |

— Not determined.

Fig. 3. 100 MHz $^1$H NMR spectrum of PR-1381 in DMSO-$d_6$.




## Biological Results

PR-1350 and PR-1381 are biologically equivalent, showing strong inhibition of a variety of Gram-positive and Gram-negative bacteria when assayed in standard broth dilution and agar dilution tests (Table 3).

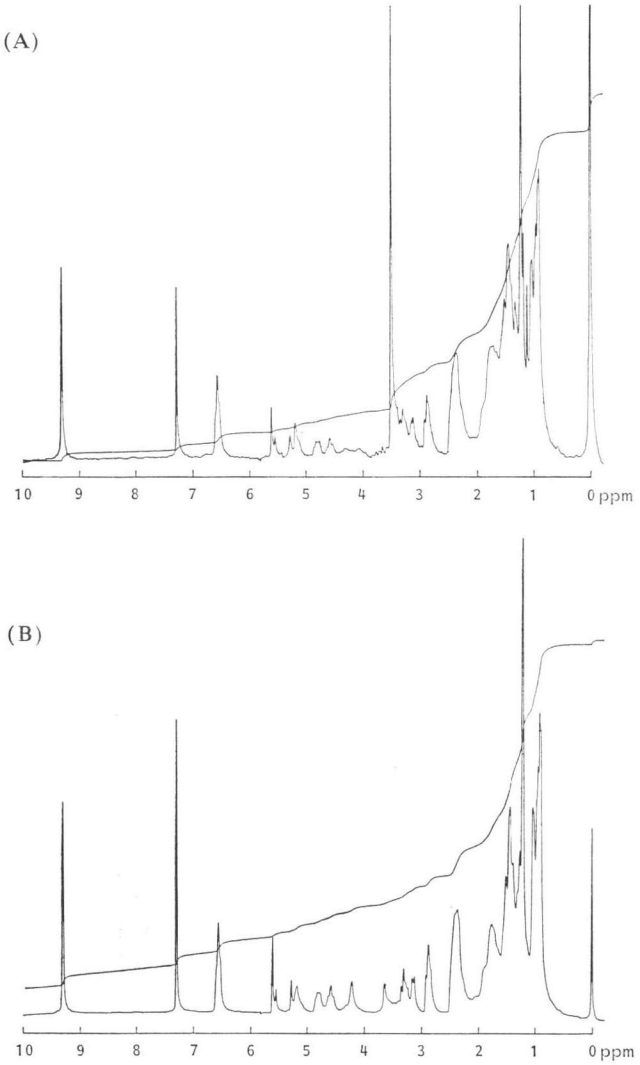
No activity was observed against a number of fungi tested in the same way.

Given intraperitoneally to mice PR-1350 has an $LD_{50}$-value of approximately 250 mg/kg with deaths occurring after about one week. The antibiotic has been tested in the P-388 lymphocytic leukemia screening model of the National Cancer Institute and has shown significant inhibition of the tumor at all doses tested (Table 4).

## Chemical Nature and Biosynthesis of PR-1350

When reduced with sodium borohydride the two forms of the antibiotic described above — the amorphous PR-1350 and the crystalline monomethanolate, PR-1381 — both give the same main pro-

Fig. 4. 100 MHz $^1H$ NMR spectra in $CDCl_3$ of PR-1381 (after 24 hours at 20°C) (A) and of PR-1350 (B).




duct, designated PR-1383. In Table 5 the $^{13}C$ NMR data for this compound are shown together with some simple deductions regarding the chemical nature of the carbon atoms. These data imply that the molecular formula of the compound is $C_{20}H_{34}O_5$ which is in accordance with the molecular peak seen at 354 in the field ionization mass spectrum. This formula leads to the assumption that the compound is derived from a diterpene. As two of the four double bond equivalents corresponding to the formula $C_{20}H_{34}O_5$ are occupied by a double bond and an epoxide (Table 5), the diterpene in question must be of the bicarbocyclic type. Furthermore, the fragments in Table 5 include six potential methyl groups (carbons 5, 7, 8, 17, 18, and 20).

Five bicarbocyclic diterpene skeletons containing six methyl groups are known[2~5]. Four of these (A~D) are shown in Chart 1 together with an illustration of the biosynthetic pathway leading to their formation from geranylgeraniol pyrophosphate (GP). The fifth skeleton (E) is shown in the same manner in Chart 2. The dots in these charts indicate the positions in which the carboxyl carbon atom of

Table 3. *In vitro* antimicrobial activity of antibiotic PR-1350.

| Organism | Medium | Strain[a] | MIC ($\mu$g/ml)[b] |
|---|---|---|---|
| *Staphylococcus aureus* | I | CJ | 0.1 |
| *Streptococcus pyogenes* NCTC8198 | II | EC | 0.1 |
| *S. faecalis* ATCC 8043 | I | EI3 | 0.3 |
| *Bacillus subtilis* | I | KA2 | 0.1 |
| *Neisseria gonorrhoeae* | III | DA3 | 0.3 |
| *N. meningitidis* NCTC8365 | III | DB | 0.1 |
| *Haemophilus influenzae* NCTC6489 | III | IX3 | 3 |
| *Escherichia coli* | I | HA | 3 |
| *Klebsiella aerogenes* 1082E | I | HC7 | 10 |
| *K. pneumoniae* | I | HE | 3 |
| *Enterobacter cloacae* P99 | I | HC8 | 10 |
| *Proteus vulgaris* ATCC 13315 | I | HJ | 3 |
| *P. mirabilis* | I | HJ3 | 10 |
| *Pseudomonas aeruginosa* | I | BA2 | 10 |
| *Serratia marcescens* ATCC 14756 | I | HG4 | 10 |
| *Shigella flexneri* NCTC8192 | I | HT | 3 |
| *Salmonella typhimurium* NCTC5710 | I | HL2 | 10 |
| *Mycobacterium phlei* | I | MO | 1 |
| *Candida albicans* ATCC10231 | IV | ZA | >100 |
| *Saccharomyces ellipsoideus* | IV | ZZ | >100 |
| *Aspergillus fumigatus* CBS | IV | ZM | >100 |
| *Trichophyton mentagrophytes* CBS | IV | ZO | >100 |

[a] Numbers refer to the Leo Company culture collection.
[b] Three-fold dilution end point read after 20 hours of incubation. Inoculum $10^4$ organisms per ml.
Media: I N.I.H. broth (Difco).
II N.I.H. broth with 2.5% horse serum.
III Blood-ascites agar.
IV Sabouraud.

Chart 1. Biosynthetic correlation of various types of diterpenes.

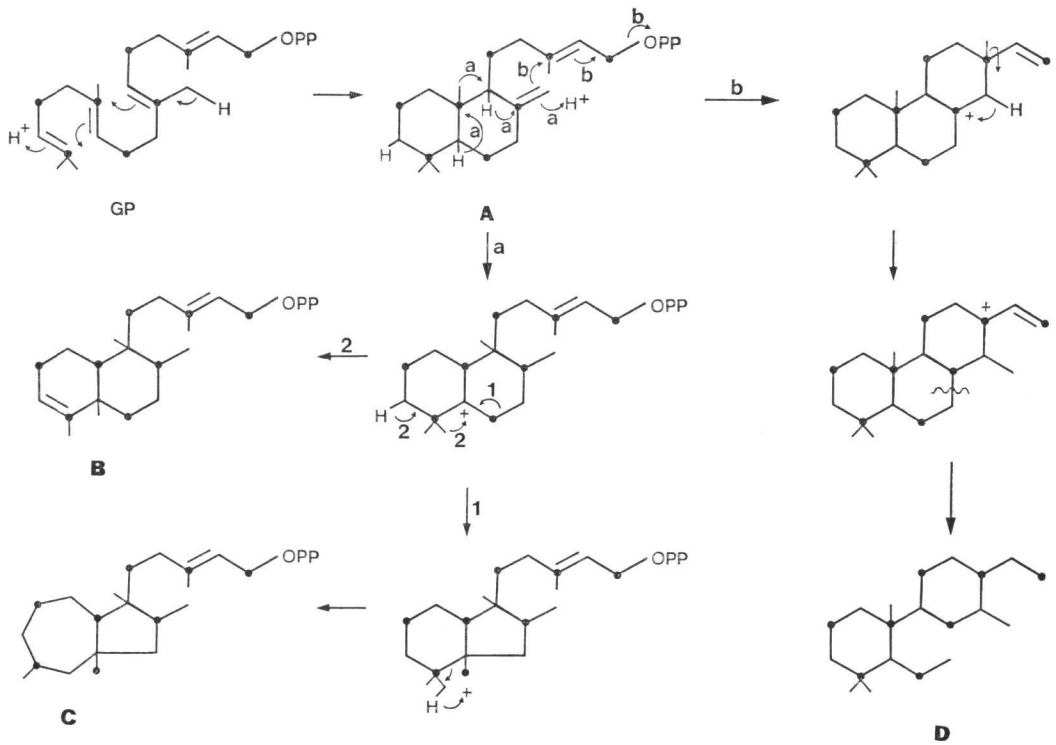


Table 4. Activity of PR-1350 against P-388 lymphocytic leukemia in the mouse (two experiments).[a]

| Dose (mg/kg)/injection | T/C[b] |
|---|---|
| 250 | 200 |
| 125 | 164 |
| 62.5 | 160 |
| 31.25 | 163 |
| 400 | — |
| 200 | 98 |
| 100 | 168 |
| 50 | 155 |
| 25 | 155 |
| 12.5 | 145 |

[a] Assays performed by the Drug Research and Development Program, Division of Cancer Treatment, National Cancer Institute. CDF mice were injected ip with $10^6$ P-388 lymphocytic leukemia cells on day 0 and treated ip on days 1~9 with the specified dose (6 mice per dose level).

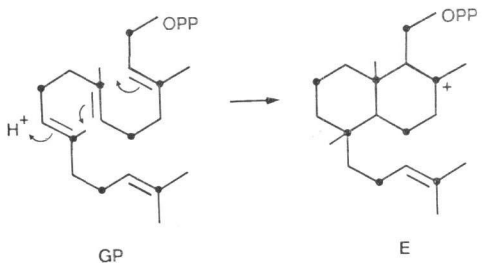
[b] T/C is the ratio of the median survival time of treated mice to that of untreated controls in per cent. Activity is defined as values of T/C≥125.

Table 5. $^{13}C$ NMR data for PR-1383 in $(CD_3)_2CO$.

| Carbon No. | Chemical shift | Multiplicity | Chemical nature | $^{13}C$-incorporation |
|---|---|---|---|---|
| 1 | 148.7 | s | >C=C< | × |
| 2 | 120.1 | d | >C=C<H | |
| 3 | 72.3 | d | >CHOH | |
| 4 | 68.9 | d | >CHOH | |
| 5 | 63.8 | t | $-CH_2OH$ | × |
| 6 | 63.1 | s |  | × |
| 7 | 62.1 | t | $-CH_2OH$ | |
| 8 | 48.7 | t |  | |
| 9 | 45.3 | d | ≥CH | × |
| 10 | 41.1 | t | $>CH_2$ | × |
| 11 | 38.7 | s | >C< | |
| 12 | 38.6 | s | >C< | |
| 13 | 37.2 | d | ≥CH | × |
| 14 | 29.8 | t | $>CH_2$ | × |
| 15 | 27.1 | t | $>CH_2$ | × |
| 16 | 26.4 | t | $>CH_2$ | |
| 17 | 22.7 | q | $-CH_3$ | |
| 18 | 21.0 | q | $-CH_3$ | |
| 19 | 19.2 | t | $>CH_2$ | |
| 20 | 16.4 | q | $-CH_3$ | |

Total: $C_{20}H_{34}O_5$

Chart 2. Biosynthesis of sacculatal diterpenes.




acetic acid is incorporated during the biosynthesis.

When PR-1383 is derived from fermentation of *O. truncatum* in the presence of sodium [1-$^{13}C$]acetate, a number of signals in the $^{13}C$ NMR spectrum show an increased intensity, as indicated in the last column of Table 5.

If PR-1383 belongs to any of the five types of diterpenes represented by the formulas A~E in Charts 1 and 2, it should be possible to fit these labelled carbon atoms into the dotted positions in that skeleton.

Three of the candidates (A, D, and E) can easily be ruled out as they all contain two labelled quaternary positions, whereas the two quaternary carbon atoms in Table 5 (11 and 12) are both unlabelled. One of the remaining skeletons (C) does indeed contain two unlabelled quaternary positions, but has to be ruled out as it contains two labelled potential methyl groups and only one such group is found in Table 5 (5). This leaves only one candidate, the clerodane skeleton (B), and inspection of Table 5 reveals that the numbers of labelled potential $CH_3$-groups, $CH_2$-groups, CH-groups, and quaternary carbon atoms found in PR-1383 (and thus in PR-1350) are exactly the same as the numbers of the corresponding dotted positions in the clerodane skeleton.

Thus, biosynthetic considerations suggest that the antibiotic PR-1350 is a diterpene of the clerodane type. This is remarkable for two reasons: Firstly, PR-1350 is to our knowledge the only known clerodane derivative showing antimicrobial activity, and secondly, this seems to be the first example of a clerodane derivative isolated from a fungus. All hitherto reported compounds of this type have been

isolated from higher plants.

The detailed structure determination of PR-1350 will be described in a forthcoming publication.

## Experimental

### General

The NMR spectra were obtained from 10% solutions (deuterated solvent Uvasole®) on a JEOL FX 100 spectrometer.

A 5 mm dual carbon/proton probe was normally used and a minimum of 16 or 2000 spectra were recorded in the case of proton and carbon, respectively.

A 70~90° single pulse was used together with a pulse repetition time sufficient to allow full relaxation. In all cases, the digital resolution was kept better than the spectrometer resolution. UV data were obtained on a Beckmann ACTA CIII spectrometer, and optical rotations were measured on a Perkin Elmer 141 polarimeter. Infrared spectra were recorded on a Perkin Elmer PE 457 grating spectrometer in KBr.

All solvents used were analytical or spectrochemical grade. Sodium [1-$^{13}$C]acetate used in the biosynthetic incorporation study was obtained from Stohler Isotope Chemicals cat. no. C80.

### Fermentation

A well-grown agar slant of strain CBS 629.70 was used to inoculate eight 2,000-ml Erlenmeyer flasks, each containing 250 ml of a medium composed of 2% corn steep liquor, 5% sucrose, 1% $KH_2PO_4 \cdot 2H_2O$ and 0.05% $MgSO_4 \cdot 7H_2O$, the pH being adjusted to 7.2 before sterilization. The flasks were incubated at 20~22°C for 3 days on a reciprocating shaker, and the growth was then transferred to eight jar fermentors each containing 5 liters of the same medium, and stirred with aeration at 20°C for 48 hours.

These seed cultures were then used to inoculate 1,500 liters of a medium composed of 2% malt extract, 2% sucrose, 0.75% soybean meal, 0.75% meat and bone meal, 0.1% $K_2HPO_4$ and 0.2% silicone (Baysilon EN-Bayer) (pH being adjusted to 6.0 prior to sterilization) in a 2,000-liters stainless steel tank fermentor operated at 20°C and 110 rpm with an aeration rate of 1,200 liters/minute, using silicone as an antifoaming agent. During the fermentation the pH was maintained at 6.0 by addition of phosphoric acid.

Samples were taken at intervals and the antibiotic activity determined by the agar cup method using *Staphylococcus aureus* as a test organism. Peak antibiotic potency was obtained after 4 days of incubation.

### Isolation of PR-1350

Fermentation broth prepared as described above was filtered using kieselguhr as a filter aid. The filtrate (1,050 liters) was passed at a rate of 2 liters/minute through a column prepared from 4.4 liters of Diaion HP-20 (height: 56 cm, diameter: 10 cm; the resin was treated before use with water, 2 N aqueous sodium hydroxide, water, methanol, 2 N aqueous hydrogen chloride, water, methanol, and water). The column was washed with water (25 liters), 25% aqueous methanol (12 liters), 50% aqueous methanol (12 liters), and 75% aqueous methanol (12 liters), and was then eluted with methanol, fractions of 2 liters being collected. Fractions 2~7, which contained the majority of the antibacterial activity, were combined and evaporated *in vacuo*. The residue was extracted with 400 ml of methanol, and after filtration the methanol was evaporated. The residue was dissolved in methanol (50 ml), and ether (800 ml) was added to the stirred solution. The precipitate formed was filtered off and discarded, and the filtrate was concentrated *in vacuo* to an oil which was dissolved in methanol - water (95: 5, 100 ml) and submitted to column chromatography on 600 ml of Diaion HP-20 (height: 90 cm, diameter: 2.9 cm) by eluting with methanol - water (95: 5). The first 500 ml of eluent were discarded and then fractions containing each 25 ml were collected. Fractions 31~103 containing about half of the activity (*S. aureus* as test organism) were combined and evaporated *in vacuo* to give 10.0 g of an oil.

This crude product was dissolved in methanol (20 ml) and applied to a column of Sephadex LH-20 (height: 90 cm, diameter: 2.9 cm), and the column was eluted with methanol. Active fractions (150 ml) were collected and evaporated to dryness *in vacuo* to yield an oil, which was dissolved in a mixture of

ether (100 ml) and cyclohexane (200 ml). The solution was treated with 3 g of Norit, filtered, and evaporated *in vacuo* to a volume of 50 ml. 200 ml of cyclohexane was added with stirring, and the precipitate which formed was filtered off, washed with cyclohexane and dried to yield 6.0 g of PR-1350 as a colorless amorphous powder.

The optical rotation of the product is given in Table 2, the IR spectrum is shown in Fig. 1, and the $^1$H NMR spectrum is shown in Fig. 4B.

The UV spectrum of the compound dissolved in ethanol shows a maximum at 230 nm ($E^{1\%}_{1cm}$ 310).

Preparation of the Crystalline Methanolate PR-1381

500 mg of amorphous PR-1350 was dissolved in 1.5 ml of methanol and the solution was left at 5°C. During 72 hours crystals slowly precipitated. 15 ml of diisopropyl ether was then added, and the crystals were collected by filtration, washed with diisopropyl ether, and dried to yield 300 mg of colorless crystalline methanolate of PR-1350, called PR-1381, mp. 139~141°C.

*Anal.* Calcd. for $C_{21}H_{32}O_6$: C 66.29, H 8.48.
Found: C 66.14, H 8.49.

The optical rotation of the product is given in Table 2, the IR spectrum is shown in Fig. 2 and the NMR spectrum of a freshly prepared solution in DMSO-$d_6$ is shown in Fig. 3.

The UV spectrum of the compound dissolved in ethanol shows a maximum at 230 ($E^{1\%}_{1cm}$ 310).

Reduction of PR-1350 with Sodium Borohydride (PR-1383)

To a solution of PR-1350 (2 g) in ethanol (100 ml) was added at 0°C sodium borohydride (4 g), and the resulting solution was stirred at 0°C for 1.5 hours. Acetone (10 ml) was then added to destroy the remaining borohydride followed after 15 minutes by ethyl acetate (800 ml) and water (200 ml). The aqueous phase was extracted twice with 200 ml of ethyl acetate, and the combined organic phases were washed with saturated aqueous sodium chloride, dried over magnesium sulfate, and evaporated *in vacuo* to dryness. From the residue the main product was isolated by column chromatography on silica gel developed with acetone - hexane (1 : 1) yielding in addition to a number of fractions in which the main product was contaminated with various by-products, 643 mg of the pure compound as a colorless oil. $^{13}$C NMR data for this product (PR-1383) are given in Table 5.

$^1$H NMR data (acetone-$d_6$, TMS as internal reference) 1.04 s, 1.17 s, 1.50 bs, 2.74 d $J$=5 Hz, 2.88 d $J$=5 Hz, 3.58 m, 3.92 m, 4.03 m, 5.49 bt.

Incorporation of Sodium [1-$^{13}$C]Acetate

A 4 liters fermentation of *O. truncatum* was performed as described above with sodium [1-$^{13}$C]-acetate (2 g) added to the fermentation medium. The antibiotic was isolated as described above to give 30 mg of amorphous material, which was reduced with sodium borohydride and purified by chromatography as described above yielding 8 mg of $^{13}$C-labelled PR-1383. The $^{13}$C NMR spectrum of this product showed that a total of eight carbon atoms were labelled with $^{13}$C as shown in detail in Table 5.

Acknowledgments

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