IMPROVED PRODUCTION OF PENTOSTATIN AND IDENTIFICATION OF FERMENTATION COMETABOLITES

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(Received for publication July 14, 1992)

A practical process is described for the large-scale isolation of pentostatin, an adenosine deaminase inhibitor used clinically for the treatment of interferon-refractory hairy cell leukemia. The identities of minor components in the fermentation beer, including 2'-deoxyguanosine, are also reported.

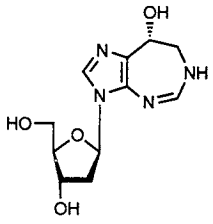
In 1969 the antiviral agent 9-(β-D-arabinofuranosyl)adenine (Ara-A, vidarabine) was isolated from the fermentation beers of *Streptomyces antibioticus*[1]. This "unnatural" adenine nucleoside is rapidly deaminated at C-6 by adenosine deaminase (ADA). Because of the large amount of Ara-A produced and the fact that ADA is commonly present in *Streptomyces* fermentation broths, chemists at Parke-Davis were prompted to search for an ADA inhibitor in *S. antibioticus* fermentation beers. These efforts were rewarded when a few milligrams of crystalline pentostatin (2'-deoxycoformycin) were isolated from several hundred liters of beer[2]. The structure of pentostain (**1**), $C_{11}H_{16}N_4O_4$, was determined by chemical and spectroscopic techniques[3] and is shown in Fig. 1. Subsequently, a total synthesis of **1**[4] and selected analogs[5] was reported. Because of its potent biological activity, the metabolism and biosynthesis of pentostatin have been extensively studied.[6~9] Additionally, pentostatin labeled either with ^3H or ^{14}C has been prepared[10,11] for pharmacokinetic studies.

Pentostatin is a powerful inhibitor of ADA and acts as a tight binding inhibitor of this enzyme[12] with a *Ki* of 2.5×10^{-12} M. As expected, pentostatin greatly enhances the antiviral activity of Ara-A[13]. In later studies, pentostatin has been found to be effective against chronic lymphocytic leukemia[14,15] and hairy cell leukemia.[16,17] Indeed, pentostatin (Nipent) is now an approved and highly effective agent for the treatment of interferon-refractory hairy cell leukemia.[18~20] To more fully evaluate pentostatin as an anticancer agent and to supply the large amounts needed for the clinic, an efficient process was required for its production.

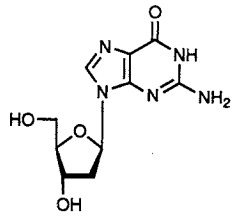
The major steps used for the original isolation of pentostatin involved a carbon adsorption/desorption procedure followed by chromatography on Darco G-60 and then on Sephadex G-10[2]. The course of the fractionation was monitored by testing fractions for their ability to inhibit the deamination of adenosine by ADA. Repeated recrystallization of the product from the final, most active chromatographic fractions afforded less than 8 g of pentostatin from 9,500 liters of beer.

The low yield and considerable labor involved in isolating pentostatin using this procedure were not practical for the production of kilogram lots of the drug. Therefore an alternative process, coupled with a rapid HPLC method for assaying pentostatin, was developed. This report describes an efficient process (Scheme 1) for the large-scale isolation of pentostatin and the identification of four cometabolites.

Fig. 1.



(1) Pentostatin†



(2) 2′-Deoxyguanosine

† (8*R*)-3-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3,6,7,8-tetrahydroimidazo[4,5-*d*][1,3]diazepin-8-ol

Scheme 1. Isolation of pentostatin.

Fermentation beer

↓ adjust to pH 8.7
store overnight at ≤15°C
add Celite 545 and filter

Beer filtrate

↓ adjust to pH 6
Dowex-50 (NH_4^+)
wash resin with H_2O
elute with 0.1～0.2 M NH_3

NH_3 eluate

↓ concentrate *in vacuo*
add EtOH-acetone (2:1)
concentrate supernatant
apply to Purolite A-425 (borate)
elute with H_2O

H_2O eluate

↓ apply to Sepabeads SP-207 column
wash with H_2O; elute with H_2O-EtOH (9:1)
concentrate, and decolorize with Norit SX2
crystallize ×2 from MeOH-H_2O (9:1)

Pentostatin (>99.7% pure)

Filtration of the harvested fermentation beer, adjusted to pH 8.7 and cooled overnight at ≤15°C, removed more than 80% of Ara-A, which is only sparingly soluble in water. The filtrate was then passed over a Dowex-50 (NH_4^+) ion exchange column, which was then washed with water and eluted with a dilute solution of aqueous NH_3 to remove the weakly basic pentostatin. Trace amounts of coformycin (2′-hydroxypentostatin) were also present in the Dowex-50 eluate. When the aqueous NH_3 eluate was concentrated and treated with a mixture of ethanol and acetone (2:1), a large amount of dark oily impurities was precipitated. The supernatant liquid was concentrated and passed over the borate form of a strong anion exchange resin such as Dowex-1 or an equivalent resin such as Purolite A-425. Several contaminants, including coformycin, were retained on the column whereas pentostatin and a small amount of cytosine were present in the early water eluates. Subsequent elution with H_2O-MeOH (9:1 then 8:2) stripped off Ara-A and further elution with MeOH-H_2O (1:1 then 3:1) afforded a fraction from which a crystalline compound was isolated. This metabolite proved to be 2′-deoxyguanosine (**2**) on the basis of spectral and chromatographic comparisons with an authentic sample.

Pentostatin from the early water eluates of the above anion exchange resin was adsorbed on a column containing a polystyrene-divinylbenzene type resin such as Sepabeads SP-207 (Mitsubishi Chemical Industries). A preliminary wash of the resin with H_2O contained a small amount of cytosine, identified by UV and HPLC comparisons with an authentic sample. Elution of the Sepabeads resin with H_2O-EtOH (9:1 w/w) afforded fractions from which pentostatin of >85% purity was recovered. The presence of a small amount of the (8*S*)-isomer of pentostatin (an artifact) was confirmed by preparative HPLC isolation and further identified by NMR spectroscopy. Treatment of the 85% pure product with charcoal followed by two crystallizations from aqueous MeOH afforded pentostatin that was >99.7% pure by HPLC. In repetitive runs, HPLC assays of the concentrate following the EtOH-acetone precipitation step showed that the overall recovery of pentostatin varied between 40～60%.

In conclusion, we have developed an efficient scale-up process suitable for isolating kilogram quantities of pentostatin for clinical use in the treatment of hairy cell leukemia and for potential application in other

indications.

Experimental

All isolation and purification steps were monitored by high pressure liquid chromatography using a 250×4.6 mm Phenomenex C8, 5 μm column; mobile phase: 0.05 M $(NH_4)_2HPO_4$ - MeOH - MeCN (95 : 2.5 : 2.5) adjusted to pH 7.4 with phosphoric acid; flow rate: 1.5 ml/minute; detection: 258 nm. In a typical run the retention times for pentostatin, cytosine, coformycin, (8*S*)-pentostatin, 2′-deoxyguanosine, and Ara-A were: 6.9, 2.3, 4.8, 5.1, 6.1, and 9.1 minutes, respectively. During some of the column runs the percentage of MeOH in the mobile phase was increased to 4% ~ 6% so that the retention time for pentostatin was reduced to about 2.5 minutes. Occasionally, TLC on silica gel plates was used to monitor certain fractionations. A suitable system is MeCN - 0.2 M NH_4Cl (3 : 1) in which pentostatin, visualized with UV light at 254 nm, has an Rf of 0.35. For analytical monitoring, a preferred system is $CHCl_3$ - MeOH - 0.5 M NaOAc (5 : 4 : 1), pH 5.0, using silica gel plates prewashed with MeOH. The Rf value for pentostatin in this system is 0.53.

Production lots of *Streptomyces antibioticus* NRRL 3238 fermentation beer containing pentostatin were prepared as previously described[21]. In a typical run 50,000 liters of fermentation beer was adjusted to pH 8.7, stored at or below 15°C for about 20 hours, and then filtered using Celite 545. The cold filtrate was adjusted to pH 6.0 and passed through a column containing 5,000 liters of Dowex-50X4 (NH_4^+) previously conditioned with cold water. The Dowex-50 resin was then washed with water and eluted with 0.1 M ammonium hydroxide followed by 0.2 M ammonium hydroxide. Throughout this elution step and subsequent isolation and purification steps the course of the operation was monitored by HPLC as described above. A small amount of coformycin, identified by UV spectral and HPLC comparisons with an authentic sample, was present in the early ammonium hydroxide eluates. The eluates (approximately 2,000 liters) containing the major quantity of pentostatin were pooled and concentrated *in vacuo* to 480 liters at a temperature less than 50°C.

A 100-liter aliquot of the above concentrate was further concentrated to 50 liters and then stirred with 500 liters of 5% MeOH - 95% EtOH (3A ethanol). Acetone (250 liters) was added and the mixture was stored overnight at 5°C. The supernatant solution was carefully removed from a dark, tarry residue, filtered through Celite 545, and concentrated *in vacuo* to 20 liters at a temperature less than 28°C. The dark, viscous concentrate was applied to a 30×300 cm column containing 135 liters of Purolite A-425 (borate) resin (at least 0.10 liters/g pentostatin), prepared by passing 15 bed volumes of 0.7 M $K_2B_4O_7$ solution over the chloride form of Purolite A-425 followed by a thorough wash with deionized water. A great deal of dark color was adsorbed on the resin as the 20-liter charge passed through the column. The resin was then eluted with 7 bed volumes of deionized water. After a forerun of about one bed volume the pH of the eluate abruptly increased. About 90% of the pentostatin applied to the resin was eluted in the next 3 bed volumes. Subsequent elution with H_2O - MeOH (8 : 2) afforded fractions containing Ara-A, identified by UV spectral and HPLC comparisons with an authentic sample. Finally, elution of the resin with H_2O - MeOH (6 : 4) afforded fractions containing a compound with a retention time of 6.1 minutes. A 1.5 g portion of the residue from these fractions was chromatographed on 500 g of 8 μm C-18 silica gel, packed in a SS Dynamax column (Rainin Instrument Co.). The eluent was 0.025 M $(NH_4)_2HPO_4$ - MeCN (95 : 5), adjusted to pH 6.5 with H_3PO_4 and the course of the chromatography was monitored at 368 nm. The metabolite corresponding to the 6.1-minute component appeared after two bed volume (400 ml) of eluate had been collected. The ensuing two 50 ml fractions, which contained the majority of the 6.1-minute component, were combined, concentrated, and applied to a column containing 300 ml of Sepabeads SP-207. After washing with 500 ml of water, elution with 600 ml of H_2O - MeOH (1 : 1) gave a fraction that was concentrated to give 0.25 g of residue. Crystallization of this material from 10 ml MeOH afforded 0.18 g of product that was recrystallized from water. This compound was identified as 2′-deoxyguanosine[22] on the basis of NMR, MS, UV, IR, and chromatographic comparisons with a commercial sample (Aldrich, #85-499-9).

Pentostatin-rich fractions from the 135-liter Purolite A-425 column were pooled and applied to a 30×300 cm column containing 120 liters of Sepabeads SP-207 (about 0.12 liters/g pentostatin).

The resin had previously been washed with aqueous ethanol followed by a thorough wash with deionized water. After all of the pentostatin change had been applied, the Sepabeads resin was washed with 3~4 bed volumes of deionized water to remove a small amount of cytosine, identified by UV and HPLC comparisons with an authentic sample. The resin was then eluted with H_2O-EtOH (9:1 w/w). After a forerun of one bed volume, pentostatin began to elute from the column and was essentially completely eluted in the next 2.5 to 3.0 bed volumes. The fractions containing the major amount of pentostatin were pooled and concentrated *in vacuo* to give a 12.5% w/w solution of pentostatin. This concentrate was treated with charcoal (5% Norit SX2 w/w with respect to pentostatin) and then filtered using Celite 545. If the bulk drug was destined for clinical use, the filtrate at this stage was passed through 0.45 μm and 0.22 μm membrane filters to reduce bioburden.

The final filtrate was concentrated *in vacuo* to a slurry containing 60% pentostatin (w/w) to which 10 volumes (based on the calculated water content) of methanol was added. The resulting suspension was stirred at 0°C for 2 hours and the solid product was collected by filtration, washed with methanol, and dried at 50°C for 24 hours. HPLC analysis showed the product to be pentostatin contaminated with 1.1% of other UV-absorbing compounds. The major contaminant (retention time of 5.1 minutes) was shown to be the (8*S*)-isomer of pentostatin, an artifact[2,3] formed duirng the isolation process.

This first crop of crystalline pentostatin was dissolved in 1.2~1.5 parts of hot (82°C) water, then with stirring the solution was diluted with 10 volumes of methanol. The resulting mixture was allowed to cool to 25°C, chilled at 0°C for 2 hours and then filtered. The collected solid was washed with methanol and dried at 50°C *in vacuo* to give 648.5 g pentostatin, >99.7% pure by HPLC, and representing approximately a 60% recovery of the pentostatin in the concentrated Dowex-50 eluate. The final product, mp 212~214°C (dec), displayed UV, IR, and NMR spectra identical to the corresponding spectra previously reported for pentostatin.[2,4]

Acknowledgments

We thank the Ann Arbor Pilot Plant Group, headed by Mr. NEIL WILLMER, for the early development of the large scale isolation steps and the Pontypool Group, headed by Drs. T. G. GOODBURM and J. B. GARNER for the final large scale isolation of pentostatin. Analytical support in Pontypool was provided by Dr. K. J. CALDICOTT's group. This project was supported in part by contract NO1-CM-37614 granted to Warner-Lambert by the National Cancer Institute, U.S.A.

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