NEW MACROLIDES FROM *PENICILLIUM URTICAE* MUTANT S11R59

DARARAT RODPHAYA, JUNICHI SEKIGUCHI† and YASUHIRO YAMADA

Department of Fermentation Technology, Faculty of Engineering, Osaka University, Yamada-oka 2-1, Suita-shi, Osaka 565, Japan
†Department of Applied Biological Science, Faculty of Textile, Shinshu University, Tokida 3-15-1, Ueda-shi 386, Japan

(Received for publication October 9, 1985)

Two new macrolides, patulolide B and patulolide C, were isolated from a culture filtrate of *Penicillium urticae* S11R59 mutant. The structures of these macrolides were determined and their biological activities were investigated. These structures and biological activities were also compared with those of patulolide A which was produced by the same organism.

In the course of studying the biosynthesis route of patulin using *Penicillium urticae* NRRL 2159A strain, several patulin-minus mutants were isolated[1]. One of these mutants, *P. urticae* S11R59, was found to produce a new macrolide in its culture broth which we named patulolide A (**I**)[2] and its structure was shown in Fig. 1. Patulolide A is a 12-membered macrolide which has a double bond flanked with carbonyl groups. It belongs to a rather new group of simple macrolides which contain cladosporlide A (**IV**), B (**V**), produced by *Cladosporium cladosporioides*[3~6] and recifeiolide (**VI**) produced by *C. recifei*[7].

In the course of studying biosynthesis of patulolide A by *P. urticae*, we analyzed culture broth of *P. urticae* S11R59 and found two new macrolides which we named patulolide B (**II**) and patulolide C (**III**). Patulolide B was not found when the strain was grown in glucose - yeast extract medium used for patulolide A production in previous study[2]. They turned out to be a congeners of patulolide A. In this report, we describe the structure elucidation of patulolides B and C and biological activities of these macrolides compared with patulolide A.

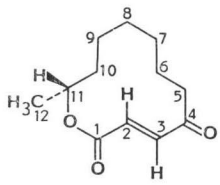
Cultivation

P. urticae S11R59, a patulin and adenine minus strain[2], was grown in two Sakaguchi flasks (2 liters) containing 500 ml of seed culture medium which contained glucose 2%, maltose 3%, raw soy bean powder 1.5%, corn steep liquor 1.0%, Polypepton (Daigo Eiyo Kagaku, Japan) 0.3% and NaCl 0.3% (pH 6.0). These flasks were cultivated on a reciprocal shaker at 28°C for 24 hours. These two flasks of seed culture were inoculated in 100 liters of production medium which consisted of seed culture medium supplemented with 0.05% antifoaming agent Actocol (Takeda Chem. Ind. Ltd., Japan). The inoculated medium placed in 200 liters fermentor was cultivated at 28°C with agitation speed of 180 rpm and aeration rate of 1 v/v/minute for 48 hours.

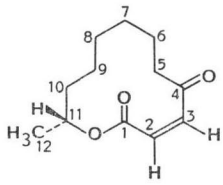
Purification

Culture filtrate of 200 liters culture broth was extracted with ethyl acetate (1/3 volume) 2 times at pH 6~7 and the extract was washed with 2% $NaHCO_3$ solution and water. The evaporation of ethyl acetate gave 71 g of crude oil. Crude extract (71 g) was applied onto 300 g of silica gel column (70~230 mesh Merck) and was roughly fractionated into 5 portions by using dichloromethane. Frac-

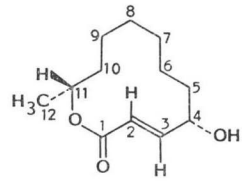
Fig. 1. Patulolides and related 12-membered ring macrolides.



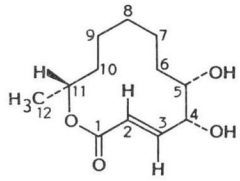
Patulolide A (**I**)



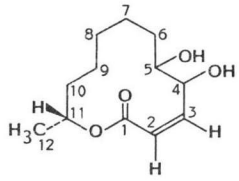
Patulolide B (**II**)



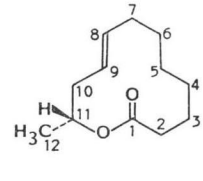
Patulolide C (**III**)



Cladospolide A (**IV**)



Cladospolide B (**V**)



Recifeiolide (**VI**)

tions No. 3, 4 and 5 which contained the mixture of patulolides A, B and C were combined to give 39.1 g of crude oil. This mixture was rechromatographed on Prep PAK-500 silica gel column of preparative HPLC (Prep LC/System 500, Waters Associates) using *n*-hexane - ethyl acetate (85: 15) as solvent system with flow rate of 100 ml/minute. Patulolide B was obtained from 1.3 liters to 1.7 liters of elution. Patulolides A and B were obtained in the yield of 3.61 g and 6.39 g, respectively. Crude patulolide C was eluted out at 3.0 liters to 4.2 liters of elution giving 8.6 g of patulolide C mixture. All patulolide C mixture was applied again onto preparative HPLC (System 500) and eluted with *n*-hexane - isopropyl ether - 2-propanol (8: 2: 1) solvent system, giving 6.29 g of pure patulolide C.

Structure of Patulolide B

The IR and ^{13}C NMR spectra of patulolide B showed that it has the same functional groups as patulolide A[2] such as one ketone group, one double bond and one ester group. The molecular ion peak (*m/z* 210) of patulolide B in mass spectrum and elementary analysis of it indicated that it has the same molecular formula with patulolide A. The value of specific optical rotation of patulolide B is minus which is opposite to that of patulolide A. ^1H NMR pattern of patulolide B was also similar to that of patulolide A and there is a coupling between the proton signals at δ 1.30 and 4.95 which indicates the presence of CH_3–CH–O structure. Coupling constant between olefinic protons at δ 6.45 and 5.97 is 12 Hz which is smaller than the value (16 Hz) of patulolide A. This fact suggests *cis* geometrical isomerism at the double bond. Hydrogenation of patulolide A and patulolide B on Pd-C in methanol gave the same dihydropatulolide which had ^1H NMR and IR spectra identical to each other. Thus the absolute configuration of C-11 in patulolide B was determined to be *R*. On the basis of these results, the structure of patulolide B was assigned as **II** (Fig. 1) which is a geometrical isomer of patulolide A.

Structure of Patulolide C

The IR, ^{13}C NMR and ^1H NMR spectra of patulolide C showed that it has one lactone carbonyl group, one double bond and one hydroxyl group. The molecular ion peak of patulolide C was *m/z*

Fig. 2. Oxidation of patulolide C to patulolide A.

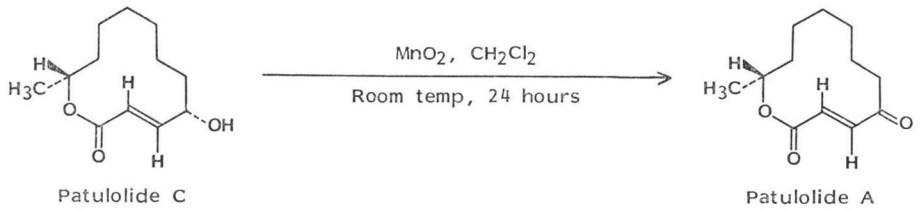


Fig. 3. X-Ray crystallographic structure of patulolide C *p*-bromobenzoyl ester.

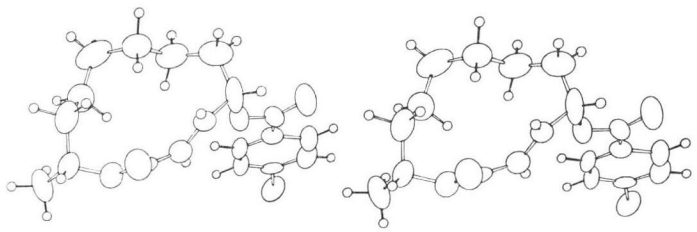


Table 1. Antifungal spectrum of patulolides A, B and C.

Test organisms	MIC (μg/ml)		
	Patulolide A	Patulolide B	Patulolide C
Alternaria kikuchiana IFO 7515	25	50	>100
A. mali IFO 8984	25	50	>100
Botryotinia fuckeliana IFO 7293	50	50	100
Cladosporium cucumerinum IFO 6370	25	50	100
Cochliobolus miyabeanus IFO 5277	100	50	>100
Diaporthe citri IFO 9170	3.1	12.5	50
Glomerella cingulata IFO 6459	25	50	>100
Helminthosporium sigmoideum var. *irregulare* IFO 5273	25	50	100
Rhizoctonia solani KHG-2	6.2	12.5	50
Pyricularia oryzae IFO 5279	12.5	12.5	50
Venturia pirina IFO 6189	25	12.5	100
Penicillium chrysogenum IFO 4626	50	25	>100
Aspergillus niger IFO 4066	100	50	>100
A. fumigatus IFO 6344	100	50	100
Trichophyton mentagrophytes IFO 7522	12.5	25	50
Microsporum gypseum IFO 6078	25	25	100
Sporothrix schenchii IFO 8158	25	50	100
Candida albicans IFO 0410	50	50	>100
Cryptococcus neoformans IFO 0410	12.5	50	>100
Saccharomyces cerevisiae IFO 0209	50	50	>100

212 (M^+). ^{13}C NMR data, elementary analysis of it and its *p*-bromobenzoyl ester indicated that the molecular formula of patulolide C is $C_{12}H_{20}O_3$. The ^1H NMR pattern of patulolide C showed the coupling between the protons at δ 1.27 and 5.04 which indicates the presence of CH_3–CH–O structure as patulolide A. Since the coupling constant of olefinic protons (δ 6.85, 6.04) is 16 Hz, the geometrical isomerism of the double bond is determined to be *trans*. Oxidation of hydroxyl group of patulolide C to carbonyl group by active MnO_2 gave patulolide A and reduction of the carbonyl group of patulolide A with (*tert*-BuO)$_3$LiAlH led to epipatulolide C. Consequently the structure of patulolide C was

Table 2. Antibacterial spectrum of patulolides A, B and C.

Test organisms	MIC (μg/ml)		
	Patulolide A	Patulolide B	Patulolide C
Escherichia coli LD-2	>100	25	>100
E. coli CPC 20	100	12.5	>100
E. coli PG 12	25	3.13	>100
E. coli PG 8	>100	25	>100
E. coli PG 8S	25	3.13	100
Citrobacter freundii IFO 12681	>100	25	>100
Klebsiella pneumoniae IFO 3317	>100	50	>100
Staphylococcus aureus FDA 209 P	50	>100	>100
Micrococcus luteus IFO 12708	100	>100	>100
Bacillus subtilis NIHJ PcI 219	100	>100	>100
B. cereus FDA 5	50	100	>100
B. megaterium IFO 12108	100	>100	>100
Brevibacterium thiogenitalis ATCC 19240	100	>100	>100

correlated with that of A. These facts clearly showed that the location of hydroxyl group is at C-4 and the absolute configuration of C-11 is *R* as patulolides A and B. The configuration at C-4 was determined to be *S* by the X-ray analysis of patulolide C *p*-bromobenzoyl ester. It is interesting that reduction of patulolide A with (*tert*-BuO)$_3$LiAlH stereo-specifically gave one configuration which corresponds to that of epipatulolide C. On the basis of these results, the structure of patulolide C was assigned as **III** in Fig. 1. Patulolide B was not detected in the culture broth of glucose - yeast extract medium from which patulolide A was first isolated. It is very interesting that the production of *cis* and *trans* isomers depends upon the culture conditions and the relationship between these three congeners on the biosynthetic pathway of secondary metabolites in *P. urticae* also attracts our attention.

Antifungal and Antibacterial Activities of Patulolides A, B and C

Antifungal activities of patulolides A, B and C were shown in Table 1. Patulolides A and B inhibit various strains of fungi and yeasts and their effects are clearly more potent than that of patulolide C. Patulolide C possesses the weakest inhibition effect to both fungi and yeasts. Yeasts, compared with fungi, are more sensitive to patulolides A and B. Among patulolides A and B we cannot say clearly which one possesses stronger inhibitory activity to fungi or yeasts from our present data. Patulolide C showed no antibacterial activity as shown in Table 2 while patulolides A and B have some activities to both Gram-positive and Gram-negative bacteria. Patulolide A possesses higher antibacterial activity against Gram-positive bacteria than patulolide B while patulolide B showed higher activity against enteric-bacteria than patulolide A. Patulolides A, B and C did not show any inhibitory effect to other Gram-negative bacteria such as *Serratia marcescens* IFO 12648, *Proteus mirabilis* ATCC 21100, *P. vulgaris* IFO 3988, *Pseudomonas aeruginosa* IFO 3080 and C 141, *Acinetobacter calcoaceticus* IFO 12552 (MIC >100 μg/ml). From the results of these antimicrobial activities and the structures of these macrolides, the essential structure for the activity is the double bond flanked with carbonyl groups which are common in other antimicrobial agents such as pyrenophorin[8,9], pyrenolides[10,11], vermiculine[12] and A26771B[13].

Experimental

Analytical Methods

^{1}H NMR spectra were obtained with Hitachi R-24B (60 MHz) or a Jeol model PS100 (100 MHz)

spectrometer. ^{13}C NMR spectra were obtained with a Jeol model SX 605 (60 MHz) spectrometer. Chemical shifts were recorded in ppm against tetramethylsilane as internal standard. UV spectra were measured on double beam spectrometer, Hitachi 557. Optical rotation was measured with a Jasco DIP-181 digital polarimeter. Mass spectra were obtained with Hitachi MRU-6E spectrometer. IR spectra were obtained by Hitachi 215 grating infrared spectrometer or Hitachi 285 infrared spectrometer.

Physico-chemical Properties of Patulolides B and C

Patulolide B: MP 66~67°C. *Anal* Calcd for $C_{12}H_{18}O_3$: C 68.54, H 8.63. Found: C 68.35, H 8.47. Mass *m/z* 210 (M^+), 192, 166, 137, 114, 96, 83, 69, 55. Optical rotation $[\alpha]_D^{25}$ −42.36° (*c* 2, EtOH). UV λ_{max}^{MeOH} nm (ε) 207 (8,085). IR (KBr) cm^{-1} 3070, 2960, 2870, 1740, 1720, 1625, 1385, 1250, 1180, 1135, 1080, 1030, 980. ^1H NMR (100 MHz, $CDCl_3$) δ 6.45 (1H, d, *J*=12 Hz, olefinic proton), 5.97 (1H, d, *J*=12 Hz, olefinic proton), 4.95 (1H, m, CH–O), 2.65 (2H, t, *J*=12 Hz, CH_2CO), 1.96~1.12 (10H, m, CH_2), 1.3 (3H, d, *J*=6 Hz, CH_3). ^{13}C NMR (60 Hz, $CDCl_3$) δ 19.63 (q, CH_3), 20.38, 23.34, 24.31, 24.90, 31.89 (t, CH_2), 40.23 (t, CH_2CO), 74.54 (d, CH–O), 125.90, 139.67 (d, =CH), 165.27 (s, COO), 202.81 (s, C=O).

Patulolide C: *Anal* Calcd for $C_{12}H_{20}O_3$: C 67.89, H 9.50. Found: C 66.98, H 9.59. Mass *m/z* 212 (M^+), 194, 167, 111, 102, 84, 83, 81, 69, 55. Optical rotation $[\alpha]_D^{25}$ −1.89° (*c* 2, EtOH). UV λ_{max}^{MeOH} nm (ε) 212 (9,169). IR (KBr) cm^{-1} 3440, 2930, 2860, 1700, 1640, 1440, 1350, 1265, 1150, 985, 870. ^1H NMR (100 Hz, $CDCl_3$) δ 6.85 (1H, dd, *J*=8, 16 Hz, olefinic proton), 6.04 (1H, d, *J*=16 Hz, olefinic proton), 5.04 (1H, m, CH–O), 4.43 (1H, m, CH–O), 3.1 (1H, s, OH), 1.92~0.80 (12H, m, CH_2), 1.24 (3H, d, *J*=8 Hz, CH_3). ^{13}C NMR (60 MHz, $CDCl_3$) δ 19.37 (CH_3), 20.84, 22.36, 27.74, 28.28, 33.12 (CH_2), 35.96 (CH_2CO), 70.74, 73.24 (CH–O), 121.19, 150.39 (=CH), 168.16 (COO).

p-Bromobenzoyl Ester of Patulolide C: MP 134~135°C. *Anal* Calcd for $C_{19}H_{25}O_4Br$: C 57.58, H 6.10, Br 20.16. Found: C 57.38, H 5.81, Br 19.86. ^1H NMR (100 MHz, $CDCl_3$) δ 7.92, 7.60 (2H, dd, aromatic protons), 6.92 (1H, dd, *J*=16, 6 Hz), 6.12 (1H, d, *J*=16 Hz), 5.62 (1H, m, CH–O), 5.10 (1H, m, CH–O), 2.20~1.04 (12H, m, CH_2), 1.30 (3H, d, *J*=8 Hz, CH_3).

Hydrogenation of Patulolide B

Patulolide B (100 mg) dissolved in 50 ml of MeOH was hydrogenated on 30 mg of Pd-C (5%) under atmospheric pressure at room temperature for 12 hours. Catalyst was removed by filtration and the filtrate was concentrated to give about 100 mg of oily product. The crude oil was purified on preparative TLC (2 mm thick, Merck) using *n*-hexane - ethyl acetate (8: 2). Yield 34 mg.

Dihydropatulolide B: IR (KBr) cm^{-1} 2930, 2850, 1730, 1470, 1410, 1380, 1340, 1270, 1180. Optical rotation $[\alpha]_D^{23}$ −68.60° (*c* 1.69, EtOH). ^1H NMR (100 MHz, $CDCl_3$) δ 4.86 (1H, m, CH–O), 3.20~2.15 (6H, m, CH_2CO), 2.00~1.10 (10H, m, CH_2), 1.15 (3H, d, *J*=7 Hz, CH_3).

Hydrogenation of Patulolide A

Patulolide A (100 mg) dissolved in 50 ml of MeOH was hydrogenated and dihydropatulolide A was purified by the same procedure as patulolide B. Yield 56.2 mg. Dihydropatulolide A showed the same IR and ^1H NMR spectra with those of dihydropatulolide B. Optical rotation $[\alpha]_D^{23}$ −65.19° (*c* 2.81, EtOH).

Reduction of Patulolide A to Epipatulolide C

Patulolide A (100 mg) dissolved in 5 ml of tetrahydrofuran was reduced with 130 mg of (*tert*-BuO)$_3$-LiAlH at 0~5°C for 24 hours. After reduction, 5 ml of water was added to the reaction mixture and extracted with dichloromethane. After purification on preparative TLC (2 mm thick, Merck) using *n*-hexane - ethyl acetate (8: 2), 68 mg of reduced patulolide A was obtained. ^1H NMR and IR spectra of reduced patulolide A showed the similar spectroscopic properties with those of patulolide C. ^1H NMR (100 MHz, $CDCl_3$) δ 7.03 (1H, dd, *J*=4.5, 16 Hz), 6.04 (1H, d, *J*=16 Hz), 5.00 (1H, m, CH–O), 4.50 (1H, m, CH–O), 2.60 (1H, OH), 2.2~0.8 (12H, m, CH_2), 1.30 (3H, d, *J*=8 Hz, CH_3). Optical rotation $[\alpha]_D^{25}$ −14.4° (*c* 33.4, EtOH).

Oxidation of Patulolide C to Patulolide A

Patulolide C (200 mg) dissolved in 5 ml of dichloromethane was oxidized with 3 g of active MnO_2

at room temperature for 24 hours with stirring. After the reaction, MnO_2 was removed by filtration and the filtrate was concentrated to give crude oil. Oxidized product was purified by preparative TLC (2 mm thick, Merck) using *n*-hexane - isopropyl ether - 2-propanol (8: 2: 1). Yield 174 mg. ^1H NMR and IR spectra of oxidized patulolide C were identical with those of patulolide A. Optical rotation $[\alpha]_D^{25}$ +12.2° (*c* 20, EtOH).

Crystal Data of Patulolide C *p*-Bromobenzoyl Ester

Orthorhombic, space group C 222_1, $a=10.402(2)$, $b=15.397(5)$, $c=23.475(4)$ Å, $V=3760(2)$ Å^3, $Z=8$, $Dx=1.40$ g/cm^3. The intensity measurements were performed for $3°\leqq 2\theta \leqq 50°$ on a Rigaku AFC-5 diffractometer with MoKα radiation. The structure was solved by heavy atom method and refined[14] to an R of 0.086.

Antifungal and Antibacterial Properties of Patulolides A, B and C

Biological potential against fungi, yeasts and bacteria were carried out by using agar dilution method at 28°C. Modified PFEFFER's agar and glucose nutrient agar were used as seed medium for fungi and yeasts while yeast - extract antibiotic medium 3 supplemented with diaminopimelic acid was used for bacteria. Minimal inhibitory concentration was determined. Modified PFEFFER's medium contained glucose 3%, L-asparagine 0 2%, NH_4NO_3 0.3%, KH_2PO_4 0.1%, $MgSO_4$ 0.1%, Versonol (iron sodium ethanolethylenediamine triacetate 50%) 0.001%, agar 1.5% (pH 7). Vitamin B_1 hydrochloride 100 μg, niacin 100 μg, biotin 0.5 μg, folic acid 50 μg, vitamin B_6 hydrochloride 200 μg, PABA 50 μg and vitamin B_{12} 0.2 μg were added to 100 ml of modified medium before used. Glucose nutrient agar contained glucose 1.0%, peptone 0.5%, meat extract 0.5%, NaCl 0.5% and agar 2.0%.

Acknowledgments

The authors would like to gratefully thanks to Dr. YOSHIO NAKAO of Applied Microbiology Laboratory of Takeda Chemical Industries, Ltd. for the large scale cultivation, X-ray crystallographic analysis and biological activities assays.

References

1) SEKIGUCHI, J. & G. M. GAUCHER: Conidiogenesis and secondary metabolism in *Penicillium urticae*. Appl. Environ. Microbiol. 33: 147~158, 1977
2) SEKIGUCHI, J.; H. KURODA, Y. YAMADA & H. OKADA: Structure of patulolide A, a new macrolide from *Penicillium urticae* mutants. Tetrahedron Lett. 26: 2341~2342, 1985
3) HIROTA, A.; H. SAKAI & A. ISOGAI: New plant growth regulators, cladospolide A and B, macrolides produced by *Cladosporium cladosporioides*. Agric. Biol. Chem. 43: 731~735, 1985
4) HIROTA, A.; H. SAKAI, A. ISOGAI, Y. KITANO, T. ASHIDA, H. HIROTA & T. TAKAHASHI: Absolute stereochemistry of cladospolide A, a phytotoxic macrolide from *Cladosporium cladosporioides*. Agric. Biol. Chem. 49: 903~904, 1985
5) HIROTA, H.; A. HIROTA, H. SAKAI, A. ISOGAI & T. TAKAHASHI: Absolute stereostructure determination of cladosporide A using MTPA ester method. Bull. Chem. Soc. Jpn. 58: 2147~2148, 1985
6) HIROTA, A.; A. ISOGAI & H. SAKAI: Structure of cladospolide A, a novel macrolide from *Cladosporium fulvum*. Agric. Biol. Chem. 43: 799~800, 1985
7) VESONDER, R. F.; F. H. STODOLA, L. J. WICKENHAM, J. J. ELLIS & W. K. ROHWEDDER: 11-Hydroxy-*trans*-8-dodecenoic acid lactone, a 12-membered-ring compound from a fungus. Can. J. Chem. 49: 2029~2032, 1971
8) NOZOE, S.; K. HIRAI, K. TSUDA, K. ISHIBASHI, M. SHIRASAKA & J. F. GROVE: The structure of pyrenophorin. Tetrahedron Lett. 1965: 4675~4677, 1965
9) GOVE, J. F.: Metabolic products of *Stemphylium eadicinum*. IV. Minor products. J. Chem. Soc. (C): 1971: 2261~2263, 1971
10) NUKINA, M.; M. IKEDA & T. SASSA: Two new pyrenolides, fungal morphogenic substances produced by *Pyrenophora teres* (Diedicke) Drechsler. Agric. Biol. Chem. 44: 2761~2762, 1980
11) NUKINA, M.; T. SASSA & M. IKEDA: A new fungal morphogenic substance, pyrenolide A from *Pyrenophora teres*. Tetrahedron Lett. 21: 301~302, 1980
12) BOECKMAN, R. K., Jr.; J. FAYOS & J. CLARDY: A revised structure of vermiculine. A novel macrolide

dilactone antibiotic from *Penicillium vermiculatum*. J. Am. Chem. Soc. 96: 5954~5956, 1974

13) MICHEL, K. H.; P. V. DEMARCO & R. NAGARAJAN: The isolation and structure elucidation of macrocyclic lactone antibiotic, A26771B. J. Antibiotics 30: 571~575, 1977

14) STEWART, J. M.: The X-rays System. Technical Report TR-446 of the Computer Science Center, University of Maryland, MD, U.S.A.