106. Isolation and Structure of a New Antibiotic Related to Rubiflavin A[1])

by **Heinz Nadig** and **Urs Séquin***

Institut für Organische Chemie der Universität, St. Johanns-Ring 19, CH-4056 Basel

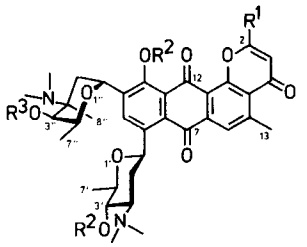
and **Richard H. Bunge, Timothy R. Hurley, David B. Murphey,** and **James C. French**

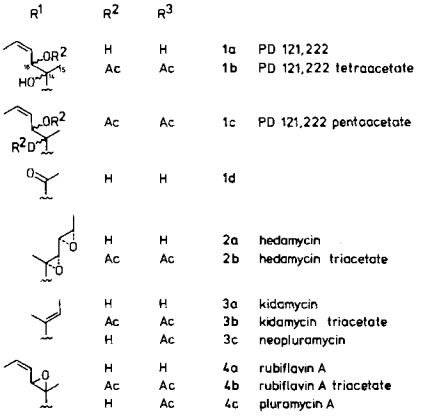
Warner-Lambert/Parke-Davis Pharmaceutical Research, 2800 Plymouth Road, Ann Arbor, MI 48105, USA

(9.IV.85)

A novel antibiotic, PD 121,222, was isolated from a complex of pluramycin-like compounds containing mostly kidamycin and neopluramycin. Spectral analyses showed that this compound is the 14,16-dihydroxy analog of rubiflavin A.

Introduction. – The family of pluramycin-like antibiotics are highly substituted 4*H*-anthra[1,2-*b*]pyran-4,7,12-triones[2]). Most of these metabolites have *C*-glycoside units attached to C(8) and C(10) [3]. One subgroup of this family, which includes hedamycin (**2a**) and pluramycin A (**4c**), has a C_6 substituent at C(2). The other subgroup, represented by kidamycin (**3a**) and neopluramycin (**3c**), bears a C_4 moiety at C(2). The newest addition to this interesting family of antitumor antibiotics is rubiflavin A which was identified as deacetylpluramycin A (**4a**) [4]. Rubiflavin A was also found to be a chromophoric constituent of the protein antibiotic, largomycin FII [5]. We now report the isolation (*cf.* details in the *Exper. Part*) and structure determination of a new pluramycin-A-type antibiotic, PD 121,222[1]), isolated from an antibiotic complex which also contained kidamycin and neopluramycin.





	R^1	R^2	R^3		
		H	H	1a	PD 121,222
		Ac	Ac	1b	PD 121,222 tetraacetate
		Ac	Ac	1c	PD 121,222 pentaacetate
		H	H	1d	
		H	H	2a	hedamycin
		Ac	Ac	2b	hedamycin triacetate
		H	H	3a	kidamycin
		Ac	Ac	3b	kidamycin triacetate
		H	Ac	3c	neopluramycin
		H	H	4a	rubiflavin A
		Ac	Ac	4b	rubiflavin A triacetate
		H	Ac	4c	pluramycin A

[1]) At *Warner-Lambert/Parke-Davis*, this new antibiotic is known as PD 121,222. Therefore, this latter designation is used throughout this paper.

[2]) The numbering in the formulae corresponds to that established for pluramycin-like antibiotics in [1] [2].

Structure Elucidation. – The IR spectra of PD 121,222 (**1a**), hedamycin (**2a**), and kidamycin (**3a**) [2] were strikingly similar; differences were observed in the region between 3600 and 3700 cm^{-1}, where PD 121,222 showed two prominent bands, whereas hedamycin and kidamycin had only one rather weak absorption. Another slight difference was observed in the region between 1600 and 1700 cm^{-1}. It is noteworthy, that PD 121,222, like hedamycin and kidamycin, did not show any ester absorption.

The ^1H- and ^{13}C-NMR spectra also were very close to those of hedamycin, kidamycin, and especially rubiflavin A [4], thus classifying PD 121,222 as being of the pluramycin-type. With the exception of the signals of the side chain at C(2), the spectra of PD 121,222 were virtually identical to those of rubiflavin A. Therefore, the phenolic OH group and the OH functions on the two tetrahydropyran rings in PD 121,222 are not acetylated. This was corroborated by the absence of any ester carbonyl band in the IR. In the ^{13}C-NMR spectrum of PD 121,222, side-chain CH_3 resonances were detected at 23.5 and 13.5 ppm. Two signals at 130.2 and 127.4 ppm indicated a C=C bond. A ^{13}C-NMR spectrum recorded in $^{12}CDCl_3$ finally revealed the signal of an additional side-chain C-atom under the right hand line of the solvent at 75.8 ppm. Furthermore, the integrals indicated that the line at 71.9 ppm corresponded to 2 C-atoms, namely C(3′) and a sixth side-chain C-atom. Thus, the substituent at C(2) of PD 121,222 is similar to that of rubiflavin A with 6 C-atoms, namely two CH_3 groups, two sp^2-C-atoms and two aliphatic C-atoms, which are bound to O-atoms as indicated by their chemical shifts.

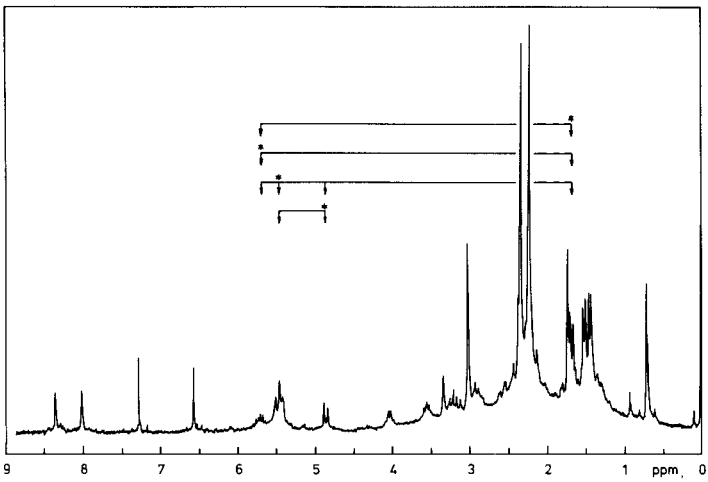
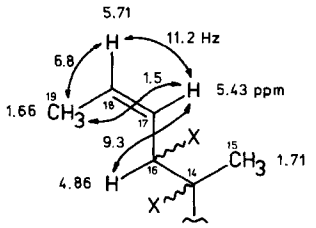


Fig. *200-MHz ^1H-NMR Spectrum of* **1a** *in $CDCl_3$.* Asterisks indicate points of irradiation in double-resonance experiments; brackets link signals of protons coupling with each other.

The ^1H-NMR spectrum of **1a** *(Fig.)*, again, was nearly identical to that of rubiflavin A, with the exception of the side-chain signals. These had slightly different chemical shifts, but displayed nearly the same coupling pattern. Two resonances (1.71 (*s*) and 1.66 ppm (*dd*, $J = 6.8$ and 1.5)) corresponded to two CH_3 groups. Further, three signals appeared at 5.71 ppm (*qd*, $J = 11.2$ and 6.8), at 5.43 ppm (partly covered by the signals of H–C(6′) and H–C(6″)), and at 4.86 ppm (*d*, $J = 9.3$ Hz). The chemical shifts of these signals and their splitting patterns, determined by homonuclear spin-decoupling experi-

ments and difference spectroscopy [6] at 200 MHz, were in agreement with the following structural element:



The (*Z*)-configuration of the double bond was indicated by the coupling constant (11.2 Hz) observed for the two vinylic protons as well as by the C-chemical shift of 13.5 ppm for the terminal CH_3 group C(19) (*cf.* rubiflavin A [4]).

A FAB mass spectrum showed a prominent fragment peak at *m/z* 679 and a signal for $(M + H)^+$ at *m/z* 749. For a pluramycin antibiotic, this latter value pointed to a molecular formula of $C_{41}H_{52}N_2O_{11}$. In antibiotics of the hedamycin/kidamycin type, where the tetrahydropyrans are not acetylated, the anthrapyran part of the molecule accounts for $C_{35}H_{41}N_2O_9$, thus leaving $C_6H_{11}O_2$ for the PD 121,222 side chain. This, together with the information gathered from the NMR spectra, leads to structure **1a** for PD 121,222. The stereochemistry of the chiral centers remains to be determined.

Chemical evidence for structure **1a** came from the fact, that PD 121,222 reacted readily at room temperature with H_5IO_6 in H_2O/dioxane. The product formed, which proved to be much less polar than the starting material (R_f 0.75 *vs.* 0.4 for **1a** on TLC with $CHCl_3/Et_3N$ 4:1), lacked the NMR signals for the side-chain protons observed for PD 121,222 at 1.66, 1.71, 4.86, 5.43, and 5.71 ppm. Instead, a CH_3 resonance at 2.88 ppm appeared. Furthermore, the H–C(3) signal was shifted to 7.02 ppm. These two resonances agree closely to the values observed (2.65 and 6.99 ppm [7]) for the corresponding protons in 2-acetylchromone. Thus, structure **1d** is assigned to the product from this HIO_4 reaction.

In addition, PD 121,222 was acetylated with Ac_2O in pyridine. Two products of distinctly different polarity were obtained (R_f 0.2 and 0.37 in $CHCl_3$/MeOH 9:1; *cf.* tri-*O*-acetylrubiflavin A: R_f 0.35). The less polar by-product could not be isolated; presumably it was the pentaacetate **1c**, where the rather hindered tertiary OH group at C(14) had also been acetylated. The more polar major product showed four Ac resonances in the ^{1}H-NMR spectrum, three of which (at 2.14, 2.19, and 2.53 ppm) corresponded to the ring E, ring F, and phenolic acetates known from tri-*O*-acetylhedamycin (**2b** [8]), tri-*O*-acetylkidamycin (**3b** [1]) and tri-*O*-acetylrubiflavin A (**4b** [4]). Typical acetylation shifts were observed for the sugar protons [1]. The additional Ac resonance at 2.10 ppm had to correspond to a side-chain acetate. The corresponding α-H-atom (H–C(16)) was shifted downfield by 1.3 ppm. These findings confirm the presence of an OH group at C(16) and corroborate the assignment of structure **1a** to PD 121,222.

Financial support of the work in Basel by the *Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung* is gratefully acknowledged. The work at *Warner-Lambert* was supported in part by contract N01-CM-07379 awarded by the *National Cancer Institute, USA.*

Experimental Part

General. UV spectra were recorded using a *Cary*, model *14* spectrometer, and IR spectra on a *Perkin-Elmer*, model *125* spectrometer. ^{1}H-NMR spectra were recorded at 200 MHz on a *Bruker WP-200 SY* spectrometer. ^{13}C-NMR spectra were recorded at 22.63 MHz on a *Bruker WH-90* spectrometer. Chemical shifts are reported in ppm downfield from internal TMS. The FAB-MS spectrum was taken on a *VG 7070E* mass spectrometer. *E. Merck* silica gel *60* plates were used for TLC.

*Isolation of 2-[(Z)-1,2-dihydroxy-1-methyl-3-pentenyl]-11-hydroxy-5-methyl-8-[3-(dimethylamino)-2,3,6-trideoxy-β-*D-arabino-*hexopyranosyl]-10-[3-(dimethylamino)-3-*C-*methyl-2,3,6-trideoxy-α-*L-lyxo-*hexopyranosyl]-4*H-*anthra[1,2-b]pyran-4,7,12-trione* (PD 121,222, **1a**). Fermentation beer (485 l), obtained by growing *Streptomyces sp. WP 0123* for 119 h at 30° in a medium containing 3% glucose, 1.0% soybean meal, 0.5% meat peptone (WP 159), 0.5% NaCl, 0.2% NH_4Cl, and 0.25% $CaCO_3$, was adjusted to pH 8.5 and stirred with 265 l of AcOEt for 45 min. *Celite 545* (23 kg) was then added and the mixture was filtered. The filter pad was washed with 150 l of AcOEt, and the wash was added to the filtrate. The layers were separated, and the lower aq. layer, readjusted to pH 8.5, was extracted with a second AcOEt wash (190 l) of the filter pad. The two AcOEt extracts were combined and extracted twice with 100 l portions of H_2O previously adjusted to pH 2.8 with H_2SO_4. Each aq. extract was quickly adjusted to pH 6.5 as soon as it was separated. The aq. extracts were combined, adjusted to pH 8.7 and extracted four times with 20-l portions of 1,2-dichloroethane. The org. extracts were combined and extracted six times with 11-l portions of H_2O adjusted to pH 2.6 with H_2SO_4. The aq. extracts were combined, adjusted to pH 6.7 and extracted three times with 18-l portions of AcOEt (remaining H_2O layer: *W-1*). The org. layers were combined, dried (Na_2SO_4), and concentrated to 60 ml. This concentrate was purified by chromatography, in two equal portions, over silica gel using a *Prep LC/system 500* instrument *(Waters Associates,* Milford, MA) The silica gel, contained in a *PrePAK-500* column, was equilibrated with $CHCl_3$/MeOH/1.3M (pH 7.3) NH_4OAc buffer 75:25:3 before application of the charge which was then purified by chromatography using $CHCl_3$/MeOH/1.3M (pH 7.3) NH_4OAc buffer 95:5:0.25. The fractions containing the majority of neopluramycin (as detected by HPLC of each fraction) from both runs were combined (4 l), washed twice with 1-l portions of H_2O, dried (Na_2SO_4), and concentrated to dryness *in vacuo*. A soln. of the residue in 17 ml of AcOEt was treated with 100 ml of cyclohexane to afford 0.69 g of *neopluramycin* (**3c**), identified by TLC, HPLC, IR, and ^{1}H-NMR comparisons with an authentic sample[3]).

The aq. layer *W-1* was extracted three times with 14-l portions of $CHCl_3$. The $CHCl_3$ extracts were combined, concentrated to 2 l, and extracted four times with 900-ml portions of H_2O previously adjusted to pH 2.8 with HCl. The acidic extracts were combined, washed with 250 ml of $CHCl_3$, then adjusted to pH 6.8 and extracted four times with 400-ml portions of $CHCl_3$ (the remaining aq. solution: *W-2*). The $CHCl_3$ extracts were combined, washed with 150 ml of H_2O, and concentrated to 12 ml. Addition of 70 ml of cyclohexane afforded 1.01 g of a product, from which 0.62 g of *kidamycin* (**3a**) (identified by TLC, HPLC, IR, and ^{1}H-NMR comparisons with an authentic sample) was isolated using a chromatographic procedure similar to that described above.

The final aq. layer *W-2* was adjusted to pH 8.3 and extracted six times with 800-ml portions of AcOEt. The org. extracts were combined, washed with 50 ml of H_2O, dried (Na_2SO_4), and concentrated *in vacuo* to 25 ml. This concentrate was treated with 30 ml of cyclohexane to precipitate 2.56 g of an orange solid (product A). TLC of this product using $CHCl_3$/MeOH/1.3M NH_4OAc adjusted to pH 9.5 with 28% NH_3 60:40:5 or $CHCl_3$/Et_3N 4:1 showed that it contained **1a**, kidamycin, a small amount of neopluramycin, and at least two other pluramycin-like components.

Product A (1.16 g) was purified by chromatography over 650 g of 40 μm *C-18* silica gel previously treated with 1 l of MeOH/H_2O 45:55 containing 25 ml of 1-pentanesulfonic acid (*PIC B5* reagent, *Waters Associates*). The MeOH content of the starting eluent, MeOH/H_2O 45:55, was gradually increased to 60% (each liter of eluent contained 25 ml of *PIC B5* reagent). Several 850-ml fractions were collected and the course of the chromatography was monitored by HPLC. *Fractions 3-9*, which contained **1a** as the only UV-absorbing component were combined, adjusted to pH 6.5, and concentrated in vacuo to remove MeOH. The remaining aq. soln. was adjusted to pH 8.1 with dil. NaOH and extracted several times with CH_2Cl_2. The org. extracts were combined, washed with H_2O, dried (Na_2SO_4), and concentrated to dryness *in vacuo*. A conc. soln. of the residue in AcOEt was treated dropwise with cyclohexane to afford 199 mg of **1a** as a deep orange solid that was more than 95% pure by HPLC.

Alternatively, **1a** can be obtained from product A by careful chromatography using *Sephadex LH-20*. A sample of product A (135 mg in 2 ml of CH_2Cl_2) was purified by chromatography over 85 g of *LH-20* which was packed as a slurry in CH_2Cl_2/CH_3OH 1:1 in a 30 × 3 cm column and then washed successively with 200 ml of

[3]) We are grateful to Dr. *Kenji Maeda,* Institute of Microbial Chemistry, Japan, for supplying us with a sample of neopluramycin.

CH_2Cl_2/CH_3OH 4:1 and 500 ml of CH_2Cl_2. Elution was carried out with 1 l of CH_2Cl_2 (25-ml fractions were collected) followed by 2.5 l of CH_2Cl_2 containing increasing amounts of CH_3OH (45-ml fractions collected). Early fractions contained kidamycin (27 mg), late fractions contained **1a** contaminated with more polar components, and intermediate fractions afforded 32 mg of **1a** as a red-orange solid that was homogeneous according to TLC.

UV (MeOH): 243 (48000), 268 (27150), 430 (8740). UV (MeOH + KOH): 253 (42700), 333 (9660), 533 (6060). IR ($CHCl_3$): 3700, 3620, 3030, 2940, 2880, 2840, 2800, 1660, 1630, 1605, 1580, 1470, 1450, 1430, 1385, 1315, 1270, 1260, 1170, 1130, 1100, 1085, 1060. ^{1}H-NMR ($CDCl_3$): 8.35 (*s*, H–C(9)); 8.02 (*s*, H–C(6)); 6.56 (*s*, H–C(3)); 5.71 (*dq*, *J* = 11.2, 6.8, H–C(18)); 5.45 (*m*, H–C(6'), H–C(6")); 5.43 (*ddd*, *J* = 11.2, 9.3, 1.5 (from spin-decoupling experiments and difference spectra), H–C(17)); 4.86 (*d*, *J* = 9.3, H–C(16)); 4.03 (br. *q*, *J* = 6.5, H–C(2")); 3.56 (*m*, H–C(2')); 3.34 (br. *s*, H–C(3")); 3.21 (*t*, *J* = 9, H–C(3')); 3.02 (*s*, 3H–C(13)); 2.88 (*m*, H–C(4')); 2.33 (*s*, $(CH_3)_2N$); 2.22 (*s*, $(CH_3)_2N$); 1.71 (*s*, 3H–C(15)); 1.66 (*dd*, *J* = 6.8, 1.5, 3H–C(19)); 1.51 (*d*, *J* = 6.4, 3H–C(7")); 1.43 (*d*, *J* = 6.4, 3H–C(7')); 0.70 (*s*, 3H–C(8")). ^{13}C-NMR ($CDCl_3$): 188.3 (C(12)); 183.1 (C(7)); 179.0 (C(4)); 170.2 (C(2)); 159.9 (C(11)); 155.8 (C(12b)); 150.1 (C(5)); 139.9 (C(8)); 138.6 (C(10)); 137.2 (C(6a)); 133.3 (C(9)); 130.2 (C(18)); 127.4 (C(17)); 126.1 and 125.8 (C(4a), C(7a) and C(6)); 118.9 (C(12a)); 116.0 (C(11a)); 110.8 (C(3)); 77.2 (C(2')); 75.6 (C(14)); 75.0 (C(6')); 71.7 (C(3') and C(16)); 70.7 (C(3")); 69.6 (C(6")); 67.4 and 67.3 (C(2") and C(4')); 57.9 (C(4")); 40.3 ($N(CH_3)_2$ in ring E); 36.8 ($N(CH_3)_2$ in ring F); 33.3 (C(5")); 28.4 (C(5')); 24.2 (C(13)); 23.5 (C(15)); 18.9 (C(7')); 17.6 (C(7")); 13.5 (C(19)); 12.6 (C(8")). ^{13}C-NMR ($^{12}CDCl_3$ containing 0.05% ^{13}C); selected resonances:

Chem. shift	Integral	Assignment	Chem. shift	Integral	Assignment
78.5	31.9	$CDCl_3$	71.9	191.0	C(3') and C(16)
77.4	149.4	C(2')	71.0	106.0	C(3")
77.1	71.0	$CDCl_3$	69.5	74.3	C(6")
75.8	136.6	$CDCl_3$ and C(14)	67.5	165.4	C(2") and C(4')
75.2	116.2	C(6')			

FAB-MS (Xe, diamylphenol matrix): 749 ($(M + H)^+$), 679. Anal. calc. for $C_{41}H_{52}N_2O_{11} \cdot H_2O$ (766.9): C 64.21, H 7.10, N 3.65; found: C 64.11, H 6.86, N 3.49.

Acetylation of **1a**. To 3 mg of **1a**, Ac_2O (20 µl) and pyridine (35 µl) were added in a screw-cap test tube. The tube was closed, the orange soln. briefly warmed up to 40° and then left in the dark at 23° for 70 h with occasional shaking. The mixture was then treated with 20 ml of aq. 10% $KHCO_3$ in small portions and the soln. extracted three times with 25 ml of $CHCl_3$. The org. layers were combined, washed with 60 ml of a sat. aq. NaCl soln., and dried (Na_2SO_4). After removal of the solvent, 3.8 mg of a yellow amorphous solid was obtained. TLC using $CHCl_3/CH_3OH$ 9:1 showed it to be a mixture of a major product (R_f 0.2) and a minor by-product (R_f 0.37). IR ($CHCl_3$): strong ester resonances at 1730 (with sh at 1750) and at 1200–1240. ^{1}H-NMR ($CDCl_3$): selected resonances at 6.15 (*d*, *J* = 9, H–C(16)); 5.75 (*dq*, *J* = 11, 6, H–C(18)); 5.48 (*m*, H–C(17)); 5.23 (*d*, *J* = 4.5, H–C(3")); 4.90 (*t*, *J* = 10, H–C(3')); 2.53 (*s*, AcO–C(11)); 2.19 (*s*, AcO–C(3')); 2.14 (*s*, AcO–C(3")); 2.10 (*s*, AcO–C(16)); 0.98 (*s*, 3H–C(8")).

Reaction of **1a** *with* H_5IO_6. H_5IO_6 (5 mg, 22 µmol) was added to a soln. of **1a** (5 mg, 6.7 µmol) in 1.2 ml of H_2O/dioxane 5:1. The resulting orange soln. was left at r.t. for 30 min, when TLC in $CHCl_3/Et_3N$ 4:1 showed that no starting material remained. After a further 30 min, the reaction was stopped by the addition of 20 ml of a 5% aq. K_2CO_3 soln. whereupon the mixture turned purple. The products were extracted into four 10-ml portions of CH_2Cl_2, which then were combined, dried (Na_2SO_4), and evaporated to yield 2.5 mg of an amorphous orange solid (R_f 0.75, $CHCl_3/Et_3N$ 4:1). IR ($CHCl_3$): 1720 (CH_3CO). ^{1}H-NMR ($CDCl_3$): selected resonances at 7.02 (*s*, H–C(3)); 2.88 (*s*, 3H–C(15)).

REFERENCES

[1] M. Furukawa, I. Hayakawa, G. Ohta, Y. Iitaka, *Tetrahedron* **1975,** *31,* 2989.

[2] U. Séquin, *Tetrahedron* **1978,** *34,* 761.

[3] For a review of the pluramycin-like antibiotics see K. Eckardt, in 'Antitumor Compounds of Natural Origin', Ed. A. Aszalos, CRC Press, Inc., Boca Raton, FL, 1981, Vol. II, p. 27.

[4] H. Nadig, U. Séquin, *Helv. Chim. Acta* **1980,** *63,* 2446; H. Nadig, Dissertation, Basel, 1982.

[5] S. Gonda, K. Byrne, P. Herber, Y. Tondeur, D. Liberato, B. Hilton, *J. Antibiot.* **1984,** *37,* 1344.

[6] G. Massiot, S. K. Kan, P. Gonord, C. Duret, *J. Am. Chem. Soc.* **1975,** *97,* 3277.

[7] A. Fredenhagen, U. Séquin, *Helv. Chim. Acta* **1983,** *66,* 586, *ibid.* **1983,** *66,* 1010.

[8] M. Zehnder, U. Séquin, H. Nadig, *Helv. Chim. Acta* **1979,** *62,* 2525.