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SYNTHESIS AND BIOLOGICAL ACTIVITIES OF SOME PEPTIDOGLYCAN MONOMER DERIVATIVES

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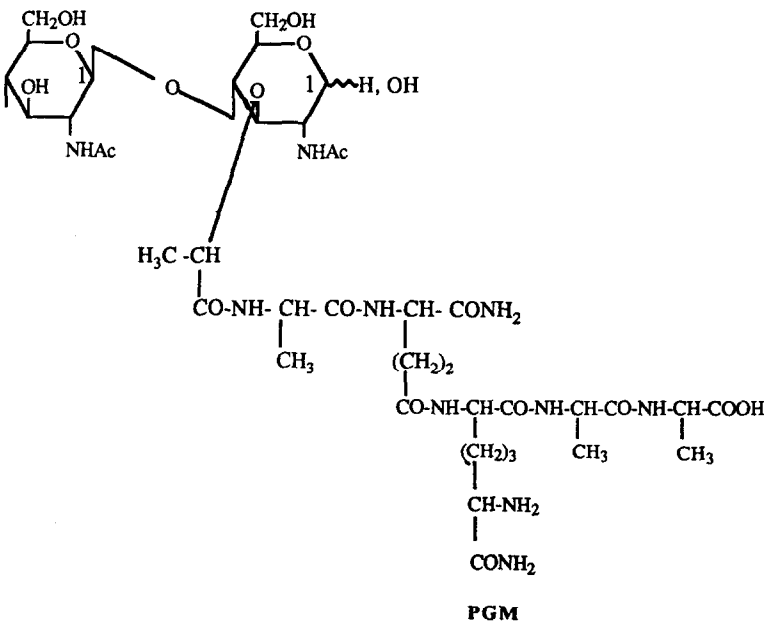
ABSTRACT. N-acyl derivatives and N-acetyl-glucosaminyl-β-(1→4)-N-acetyl-muramoyl-L-alanyl-D-isoglutaminyl-L-meso-diaminopimelyl-(D-amide)-(L)-D-alanyl-D-alanine (peptidoglycan monomer, PGM) complexes with some bivalent metals were prepared and their immunomodulatory activities examined.

INTRODUCTION

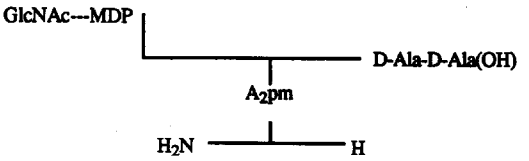
Peptidoglycans are representatives of immunobiologically active substances isolated from the cell walls of different microorganisms. Many derivatives could be obtained, depending on the microorganism, direction of the fermentation process or on the use of different enzymes for the hydrolysis of the peptidoglycanic polymers. Our peptidoglycan monomer (PGM), was isolated from *brevibacterium divaricatum* [1] and possesses a disaccharide-pentapeptide structure[2] (Fig.1).

Fig.1. Structural formula of peptidoglycan monomer



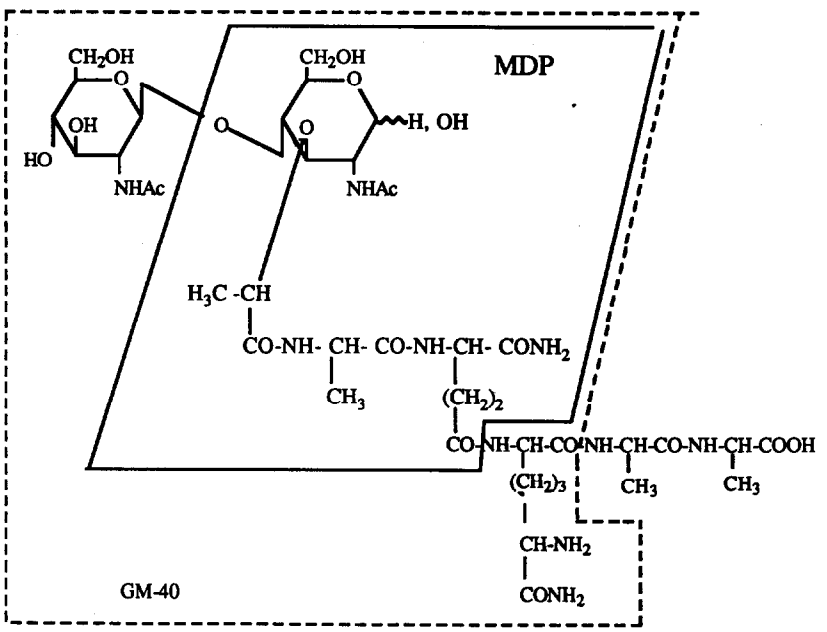
PGM

Simplified scheme of PGM



The smallest known biologically active unit with the peptidoglycanic structure has been the muramyl-dipeptide (MDP) (see Fig.2).

Fig 2. Simplified scheme of different peptidoglycan derivatives



Many other different substances with peptidoglycanic structure were isolated comprising one or two sugar units or /and with two to five amino acids in the peptide moiety of the molecule, having the same characteristic composition and arrangement as the PGM. Recently many of these substances were also obtained by chemical syntheses[3]. Except the PGM, all isolated peptidoglycans with such structures as MDP, N-acetyl-glucosaminyl-MDP-meso-diaminopimelic acid (GM-40), N-acetyl-glucosaminyl-MDP (GMDP), N-acetyl-glucosaminyl-MDP-meso-diaminopimelyl-alanine, etc. show pyrogenic effect.

Stereochemistry of the peptidic moiety of the compounds mentioned above is characterised by asymmetrical centers renewing in an order $(L\text{-}D)_n$, while only the PGM posses an order $(L\text{-}D)_n\text{-}D$ (n=2).

The PGM is soluble in water, sparingly soluble in methanol, and among the other organic solvents only soluble in N,N-dimethylformamide. Outstanding hydrophilicity and the weak lipophilic character of PGM may be modified by preparation of N-acyl derivatives. In this manner, a new equilibrium between the hydrophilic and lipophilic nature of the molecule is established which may have an influence on the immunomodulating effect by inducing a prolonged activity *in vivo* and increase affinity against target cells. Partial hydrophilic character of the molecule may be corrected also by the preparation of PGM complexes with bivalent metals, since a coordination of metal with active hydrophilic groups in PGM may enforce lypophilic parts of the molecule. This is the first attempt to prepare the metal complexes of a peptidoglycanic structure. Another reason for the preparation of bivalent metal complexes is based on the fact that metals could play an important role in the stability, distribution,

biotransformation and elimination of biological active substances. It is well known[4], that the enzyme N-acetyl-muramyl-L-alanyl-amidase, found in the serum of mammalia, splits the peptidoglycan monomer molecule into sugar and peptidic components.This enzyme is practically inactive without magnesium. It is expected that a PGM complex with metal in the molecule would be more stable in the blood-serum because the sites for the coordination with magnesium are already occupied.

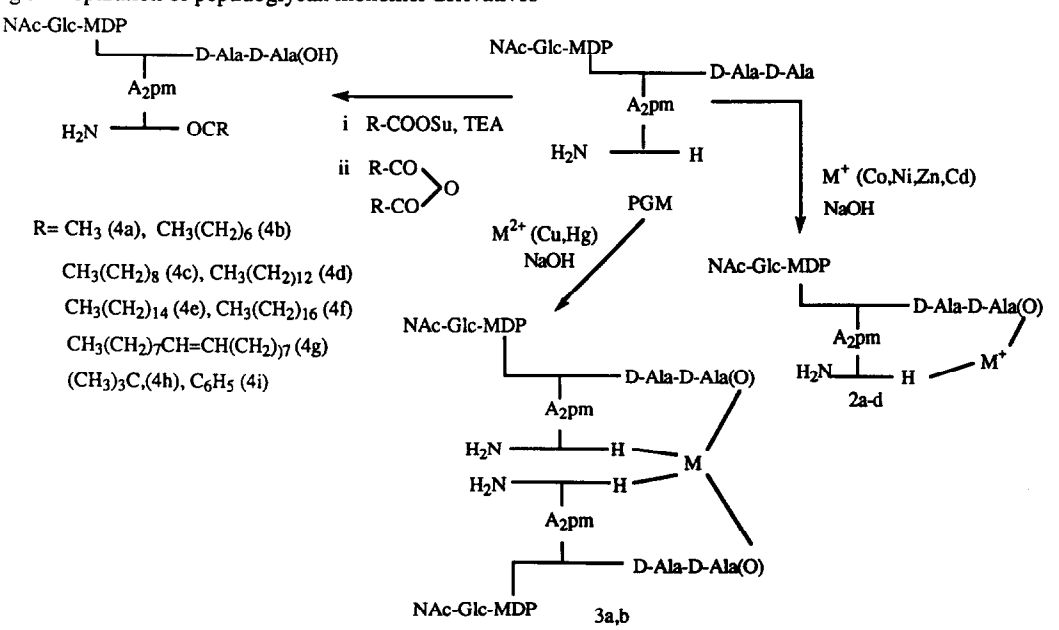
CHEMICAL PREPARATION

The process of complexing PGM with particular metal ions was followed by potentiometric titrations. The stability constants were estimated on the basis of calculation for metal amino acid complexes[5,6]. Also pH values were found where the complexes are the most stable. The metal-ligand ratio was determined by the elementary analysis and for the copper ion complex also by the Job's method of continual variations[7]. The complexes (2a-d, 3a,b) were isolated at the pH values where they were the most stable, by partial evaporation of water under reduced pressure, followed by fractional precipitation.

N-acyl derivatives (4a-i) are prepared by the usual methods of peptide chemistry. Sodium salt of N-acetyl-PGM (4a) is prepared by acetylation of PGM with acetic anhydride in aqueous solution of sodium hydrogen carbonate. The ^1H NMR spectrum of N-acetyl-PGM gave the important data for the detection of the site of acetylation in the compound (4a), indicating that the amino group is a stronger nucleophile than the terminal hydroxy group in the sugar moiety of the PGM molecule. Other N-acyl derivatives (4b-i) are obtained by condensation of the PGM with an active ester of corresponding acid. Active esters were prepared by reaction with N-hydroxysuccinimide or with N-hydroxybenztriazole and dicyclohexylcarbodiimide (DCC). Some long chain or branched alkyl carboxylic acids, unsaturated alkenyl carboxylic acid and aryl carboxylic acid are used. The acyl derivatives with long chain saturated acids after dissolution showed a tendency to gel formation. Sodium salts of corresponding N-acyl derivatives are prepared by addition of an equimolar quantity of sodium hydroxide into aqueous suspension of the N-acyl derivative, followed by lyophilization. They were purified by a column chromatography, using silicagel. In some cases preliminary purification,if is necessary, was performed on a column of Sephadex G-25 fine.

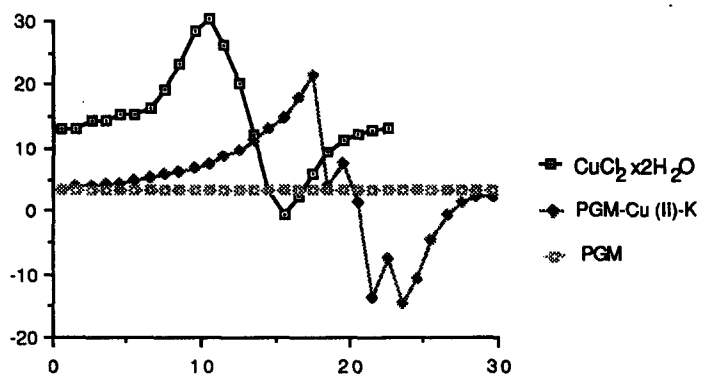
The structural scheme of pathway for the preparation of N-acyl derivatives and bivalent metal complexes of PGM is shown in Fig 3.

Fig.3. Preparation of peptidoglycan monomer derivatives



The elementary analysis of obtained PGM-complexes showed the presence of anions resulting from added salts as reagents for metal complex formations. The EPR spectrum of the copper complex (Fig.4)

Fig.4. EPR spectrum of PGM, [PGM-Cu(II)-K] and $CuCl_2x2H_2O$



confirmed the absence of PGM and metal salt mixture in the product.The site of the metal coordination in the PGM complexes is not yet definitively determined. The most useful data is obtained by the study of ^{13}C NMR spectra of the PGM-cobalt(II)-complex [PGM-Co(II)-K] and the PGM-nickel(II) complex [PGM-Ni(II)-K]. This spectra showed certain shift of the signals in comparison with the spectrum of PGM[7] (Table 1).

Table 1. Comparison of the 13C NMR spectra of PGM and [PGM-Co(II)-K]

	PGM	[PGM-Co(II)-K]
NAc-Glc		
C_1	100,4	100,4
C_2	56,05	56,0
C_3	73,6	73,6
C_4	70,45	70,2
C_5	75,35	75,4
C_6	61,25	61,2
$\underline{C}OCH_3$	173,5*	174,15*
$\underline{C}H_3CO$	22,3	22,5
NAc-Mur		
$C_1\alpha$	90,2	90,3
$C_1\beta$	95,1	–
C_2	53,7	53,6
C_3	79,5	?
C_4	76,35	76,12
C_5	71,2	71,0
C_6	59,9	60,0
$\underline{C}OCH_3$	174,0*	175,0*
$\underline{C}H_3CO$	22,0	22,2
$CH_3\underline{C}HCO$	77,45	77,43
$CH_3CH\underline{C}O$	174,9*	175,2*
$\underline{C}H_3CHCO$	18,4	18,3

continuation of Table 1.

NAc-Mur		
$C_1\alpha$	90,2	90,3
$C_1\beta$	95,1	–
C_2	53,7	53,6
C_3	79,5	?
C_4	76,35	76,12
C_5	71,2	71,0
C_6	59,9	60,0
$\underline{C}OCH_3$	174,0*	175,0*
$\underline{C}H_3CO$	22,0	22,2
$CH_3\underline{C}HCO$	77,45	77,43
$CH_3CH\underline{C}O$	174,9*	175,2*
$\underline{C}H_3CHCO$	18,4	18,3
L-Ala		
$CH\alpha$	49,6	49,4
CH_3	16,8	16,7
CO	174,6*	174,8*
D-iso-Glu		
$CONH_2$	175,2	175,4
$CH\alpha$	53,7	53,4
$CH_2\beta$	27,15	27,1
$CH_2\gamma$	31,55	31,55
CO	175,7*	175,7*
DL-meso-A2pm		
L-CO	173,5*	176,5*
$CH\alpha$	52,75	54,4•
$CH_2\beta$	30,6	32,1•
$CH_2\gamma$	20,75	26,9•
$CH_2\beta$	20,7	32,4•
$CH_2\alpha$	52,75	54,7
D-$CONH_2$	174,9	175,0
D-Ala4		
$CH\alpha$	49,6	49,8
CH3	16,75	17,83
CO	173,4*	174,8*
D-Ala5		
$CH\alpha$	49,9	49,9
CH_3	17,7	18,2•
CO	175,7*	176,0*•

These shifts are particulary significant for the signals assigned to the fifth amino acid (D-Ala5) and to diaminopimelic acid (A2pm) in the peptide moiety of the PGM molecule.The largest shift was observed for the γ

atom in A2PM moiety, what may be described to the largest change of the angle in the bond Cβ-Cγ-Cβ' (A2pm) in the complex in relation to the uncomplexed molecule. Noteworthy for the complexes is the absence of signals for the C1β-anomer (95.1 ppm) in the muraminic part of the molecule, as well as the significant shift for the C3 signal, which is not yet definitively assigned.These shifts may result from steric interaction rather than coordination with the metal.

All signals of the carbonyl groups in the complexes are upfield relative to PGM. The largest value in this shift we assigned to the carboxyl group of the terminal D-Ala5.

On the basis of this data, and contents of metal in the complexes we suggested that the coordination with metal takes place with the free amino group and with the carboxyl group and suggested that some bivalent metal ions (Co,Ni,Cd,Zn) formed complexes as showed in the formula 2a-d, and some of them (Cu,Hg) as showed in the formula 3a-b.(Fig.3).

The isolated complexes are very unstable to thin layer or column chromatography. On the silicagel plates (Merck, Kieselgel 60, F 254) using a neutral system solvent for development (for example 70% aqueous propanol), a decomposition of complexes takes place, and after visualisation only a zone of PGM was visible. Also, a partial decomposition of complexes takes place when a Sephadex G-25 fine column was used, and the metal analysis after chromatography showed a 10% loss of metal in the main peak, which was detected by the UV absorbtions.

BIOLOGICAL ACTIVITIES

The pyrogenicity test (1 mg of the substance per kg of the body mass) was made according to the method of USP XXII (the biological pyrogenicity test). All tested derivatives are proved to be apyrogenic.

The immunostimulating activity was examined by the Jerne's PFC method[9] The results are shown in Table 2 for the complexes and in Table 3 for the N-acyl derivatives.

Table 2. Immunostimulating activity of the complexes of peptidoglycan monomer with some bivalent metals [PGM-M(II)-K]

PGM-M(II)-K	% activity
Physiological solution	100
PGM	126
PGM-Cu(II)-K	143
PGM-Co(II)-K	143
PGM-Cd(II)-K	98
PGM-Zn(II)-K	121
PGM-Ni(II)-K	203
PGM + $CuCl_2x2H2O$ (2:1)	62
$CuCl_2xH_2O$	69
$NiCl_2x6H_2O$	58

Table 3. Immunostimulating activity of N-acyl PGM derivatives

Compound		%activity
Control		100
PGM		121
4a	(acetyl)	89
4b	(caproyl)	132
4c	(capriloyl)	178
4d	(lauroyl)	192
4e	(palmitoyl)	120
4f	(stearoyl)	188
4g	(oleoyl)	113
4h	(pivaloyl)	85
4i	(benzoyl)	143

It is obvious, (Table 2 and 3), that many of N-acyl derivatives (4a-h,4i) and some of complexes (2a-c,3a) show an increase in immunological response in comparison with PGM. It is also important to note that a mixture of the PGM and the copper salt in the same molar ratios as in the complex, does not exhibit any immunomodulating activity as do not also investigated inorganic salts.

EXPERIMENTAL

The IR spectra were recorded on a Perkin-Elmer Infracord Model 257 G and the UV spectra on a Pye Unicam SP8-100 instrument. The melting points were determined by Fisher-Johns apparatus and are uncorrected.The ^{1}H NMR spectra were run on a Joel 90 Q spectrometer with TMS as internal standard; chemical shifts are given in ppm-values (δ). The ^{13}C NMR spectra were recorded on a JOEL SX 90 Q spectrometer with TMS as internal standard. TLC was carried out on commercial plates (Merck, Kieselgel F_{254}). For PGM-derivatives 70% aqueous propanol was used as solvent system. Dried plates were chlorinated and spots were detected with TDM reagent [(4,4'-Bis(dimethylamino)-diphenylmethane] which is composed of a mixture of: a=0.63 g TDM/2.5 ml glacial acetic acid + 12.5 ml water, b= 1.25 g KI/25 ml water, c= 0.0285 g ninhydrin/0.95 ml glacial acetic acid + 8.55 ml water.

The following abbreviations are used:PGM= peptidoglycan monomer A_2pm= diaminopimelic acid; Ala= alanine; isoGln= isoglutamine; GlcNAc= N-acetylglucosamine; MurNAc= N-acetylmuramic acid; HOSu= N-hydroxysuccinimide; DCC= dicyclohexylcarbodiimide; EtOAc= ethyl acetate; TEA= triethylamine. PGM was produced in PLIVA-Zagreb. All inorganic salts for complexes were of analytical grade (Aldrich) and other chemicals used were of reagent grade.

Preparation of N-acyl-PGM *via* anhydride method

Preparation of sodium salt of N-acetyl-PGM (4a)

Into a solution of PGM (500 mg, 0.495 mmol) in water (7 ml) was added a saturated aqueous solution of sodium hydrogen carbonate ($NaHCO_3$ 2.75 ml) and it was cooled on an ice-bath at 5°C. Into the cooled mixture was added a dropwise acetic anhydride (2.75 ml, 29 mmol) and stirred for 24 hours at 20° C. The mixture was concentrated by evaporation at reduced pressure to yield an oily precipitate (0.95 g), which was dissolved in water (2.5 ml) and separated on a column of 150 ml of Sephadex G-25 fine, whereupon the solution was eluated with water. The product containing fractions were combined and lyophilized.
Yield 490 mg (92%).
M.p. 186-188°C; $UV\lambda_{max}(H_2O)$(nm):202; $IR^{KBr}(cm^{-1})$:
3400-3240,1660,1550,1410.

^{1}H NMR(D_2O)(ppm):1.90 (s, 3H CH_3CONH-A2pm), 1.96 (s, 3H CH_3CONH-Glc), 2.03 (s, 3H CH_3CONH-Mur)

Preparation of N-acyl-PGM *via* active ester metod

General procedure

Preparation of active esters of carboxylic acids.

A mixture of carboxylic acid (1 mmol), DCC (1.2 mmol) HOSu (1.2 mmol) and EtOAc (10 ml) was stirred at 20-24°C for 12 hours and allowed to stand over night. After filtration of the resulting precipitate, the filtrate was evaporated to dryness. The residue was applied to a column of silica gel and eluted with chloroform. Fractions containing the desired product were collected and concentrated *in vacuo* to give the N-hydroxysuccinimide ester of carboxylic acid.

Preparation of the sodium salt of N-acyl-PGM (4b-4i)

Into a solution of PGM (0.991 mmol) in DMF (20 ml) was added the active ester of carboxylic acid (1.2 mmol) and TEA (1.37 mmol) and was stirred 20 hours at 20-25 °C. As a result a gelatinous suspension was obtained into which EtOAc(135 ml)[1] under stirring was added and the stirring was continued for 5 hours.A white precipitate was formed and separated by filtration, washed with EtOAc (3 x 1 ml) and dried. The product was suspended in water, (cca 30 ml), and the pH was adjusted to 7.0[2] by adding 0.1N NaOH, and purified by means of column chromatography on Sephadex G-25 fine (elution by water) followed by silica gel (elution by 70% aqueous propanol).The product-comprising fractions were combined and concentrated by evaporation of the solvent at reduced pressure and the product was obtained by lyophilization.

Notes:

[1] In principle the EtOAc was added to end of precipitation.
[2] If at pH 7.0 a clear solution did not form, it can be increased to 7.4. If still remained turbidity, filtration was recommended.

Some physical properties of N-acyl derivatives (4b-i) are given in Table4.

Table 4. Some physical properties of N-acyl-PGM derivatives (4b-i)

N-Acyl	Mol mass (dalton)	m.p. (°C)	UV λ max (nm)	IR^{KBr} (cm^{-1})
4b	1136,2	194-195	203	3280,2930,1655,1638,1545
4c	1166,2	196-197	203	2990,1610,1580,1090
4d	1192,3	203-205	204	3280,2920,2850,1655,1543
4e	1248,4	205-208	203	3280,2920,2845,1635,1520
4f	1276,5	217-220	204	3280,2910,2350,1635,1530
4g	1246,4	204-206	198	3300,2950,2875,1640,1555
4h	1094,1	202-204	201	3400,3300,3080,3050,2980,1650, 1540
4i	1114,1	173-175	203,224 (sh)	3260,1680,1515

Preparation of metal complexes of peptidoglycan monomer

General procedure:

Peptidoglycan monomer (0.2 mmol) was dissolved in water (10 ml) and there was added a metal salt (0.2 mmol for 2a-2d, or 0.1 mmol for 3a -3b) and the solution was stirred for 1 hour. The pH was then adjusted to desired value (see table 5.) by the addition of 0.1 NaOH and the solution was stirred an additional hour. The reaction solution was concentrated by means of evaporation under reduced pressure to a volume of about 4 ml and the product was precipitated by a dropwise addition of acetone[1].
The product was filtered off, washed with acetone, followed ethanol (to negative reaction on the anion), and dried in a high vacuum for 4 hours.

Note:

[1]After addition of cca 25 ml acetone, in most cases, the oily product was formed, which after the next addition of acetone and under vigorous stirring was converted into an amorphous precipitate.

The physical and chemical properties of metal complexes of peptidoglycan monomer are shown in Table 5.

Table 5. The physical and chemical properties of metal complexes of PGM

[PGM-M(II)-K]	pH	Yield. (%)	pK_S	mp. (°C)	UVλmax (nm)	IR^{KBr} (cm^{-1})
PGM-Cu(II)-K	6.5	90	8.3	173	198,665	3420-3280,1660, 1550
PGM-Hg(II)-K	7.2	85	7.58	168	195,250	3480-3280,1675 1670,1650,1505
PGM-Co(II)-K	8.2	80	2.38	169	210,500	3430-3280,1650, 1550,1050,605
PGM-Ni(II)-K	7.5	89	3.37	176	202,645	3420-3280,1665 1550.1050
PGM-Cd(II)-K	8.2	75	2.5	170	208	3420-3280,1665 1550,1125,625
PGM-Zn(II)-K	7.5	70	2.64	171	198	3440-3260,1660,1550

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