Tetrahedron Vol 49, No 15, pp 3109-3116, 1993
Printed in Great Britain

0040-4020/93 $6 00+00
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Organochromium Complexes - Labelled Aminoglycoside Antibiotics Derived from Kanamycin A and Tobramycin. Synthesis, Structural Characterization and Use as Metallotracers for Immunoassays.

Jan Szymoniak[a*], Bouchra El Mouatassim[a], Jack Besançon[a], Claude Moïse[a] and Pierre Brossier[b]

[a]Laboratoire de Synthèse et d'Electrosynthèse Organométalliques associé au CNRS (URA 33), and

[b]Unité d'Immunoanalyse, Université de Bourgogne, BP 138, 21034 DIJON Cedex, France

(*Received in Belgium* 19 *January* 1993)

Key Words Kanamycin A , Tobramycin , Metallocenic Labels , Benchrotrene , ^{13}C NMR study , Immunoassay

Abstract *The synthesis of metallocenic derivatives of aminoglycoside antibiotics, i e kanamycin A and tobramycin, is described The organometallic-labelled compounds have been obtained from the reaction between the polyamino-drugs and organochromium esters of N-hydroxysuccinimide The reaction proceeded selectively at the 6'-N position, as might be deduced both from the mass and the pH-dependent ^{13}C-NMR spectra The procedure could be regarded as generally useful for the metallolabelling of aminoglycoside antibiotics As an example of application a competitive immunoassay based on the use of these labels is proposed*

INTRODUCTION

Labelled substances are being used more and more extensively for many applications such as pharmacological studies, clinical diagnosis, biological research, and others In particular, the metallolabelling is the first step in the development of new immunoassay methods requiring metallotracers [1] One of the most important components of non-isotopic organometallic labels is the range of analytical methods to detect them Several studies have recently been reported in which the metallic species are detected by atomic absorption,[2] electrochemically,[3] or by infrared spectroscopy in the case of metal carbonyl fragments [4]

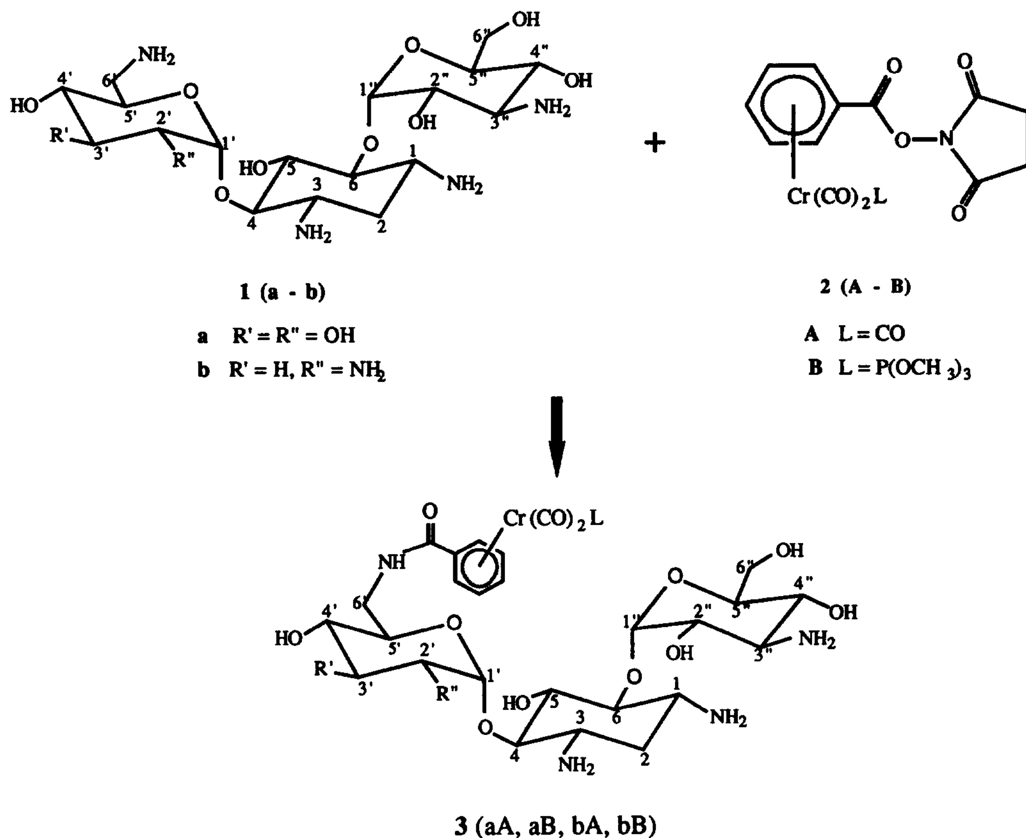
The aim of our laboratory is to synthesize therapeutic molecules labelled with an organometallic moiety as recently reported [5] In this paper we describe the labelling by chromium carbonyl fragments of a group of bactericidal drugs aminoglycosides We propose a competitive metalloimmunoassay technique[1] which use these metallolabelled compounds Indeed, the aminoglycoside antibiotics are generally difficult to analyse by chromatographic methods and there is currently an effort to develop sensitive immunoassay techniques which provide quantitative methods in the picomole range

The aminoglycoside drugs play an extremely important role in the treatment of infectious diseases and are used most widely against gram-negative bacterial infections Among the newer drugs, tobramycin, gentamycin, amikacin and kanamycin are frequently used at present. The structurally defined kanamycin A (**1a**), the major component of the antibiotic complex produced by certain strains of *streptomyces kanamyceticus*,[6] and tobramycin (**1b**)[7] have been recently studied and well characterized [8] It seemed interesting to us to try the labelling of these two drugs with the metalloimmunoassay in mind

RESULTS AND DISCUSSION

Synthesis ans Spectral Characterization

The synthesis of organochromium complexes of **1a** and **1b** has been performed by the previously described "activated ester" method [5] Two metallocenic labels used here were the N-hydroxysuccinimide esters of carboxybenzenechromium-tricarbonyl (benchrotrene) and -dicarbonylmethylphosphite, **2A** and **2B** respectively Compound **2A** was prepared by a reported procedure [5] Compound **2B** was obtained in a three step synthesis starting from methyl benchrotrenoate (see experimental section) The "activated esters" **2A, B** were then used for the selective synthesis of the 6'-N-labelled complexes **3aA, 3aB, 3bA** and **3bB** (Scheme 1)



Scheme 1

The selective nucleophilic attack of only one among the amino groups of the polyfunctional molecule **1** on the metallocenic ester **2** was achieved in the controlled, mild conditions In a typical procedure the amino drug (free base) **1** was put together with an equimolar quantity of the label **2** in a

homogenous medium, THF/water, for a period of 4-6h at room temperature in the dark TLC monitoring reveals the formation of the unique chromium-labelled compound. The not acidic conditions, and relatively short reactional period make it possible to avoid the decomposition of the drug The metallohaptens **3** were isolated with good yields as yellow or orange solids, highly soluble in water, slightly in methanol and not at all in THF (see experimental section).

The infrared characteristics of the products **3** together with starting "activated esters" **2** are summarized in Table 1

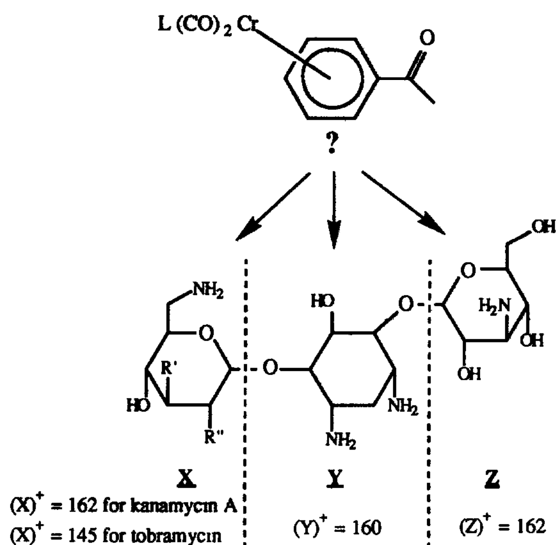
Ligand	2	3	
L = CO	**2A** 1975,1901,1778,1739	**3aA** 1970,1890,1673,1646	**3bA** 1969,1890,1673,1646
L = $P(OMe)_3$	**2B** 1931,1860,1765,1746	**3aB** 1907,1846,1676,1645	**3bB** 1906,1847,1674,1646

Table 1 Infrared data of the "activated esters" (**2**) and metallohaptens (**3**)

The absorption frequencies of the amide C=O groups of **3** appeared in the ranges 1673-6 and 1645-6 cm^{-1}. In addition, the spectra of products **3** exhibited characteristic absorptions of the chromium-tri or -dicarbonyl groups in the range 1970-1846 cm^{-1} The values of the terminal dicarbonyl absorptions in **3aB** and **3bB** were lower compared with the tricarbonyl absorptions in **3aA** and **3bA**, due to the remaining $P(OMe)_3$ ligand In accordance with the IR data, the ^1H NMR spectra of compounds **3** exhibited the typical benchrotrenic resonances at 5 6-6 2 ppm, corresponding to one organometallic unit (5 protons) incorporated

The sites of labelling of the kanamycin A (**1a**) and tobramycin (**1b**) were determined from the mass and ^{13}C NMR spectra of the resulting metallohaptens **3** The analysis of the characteristic mass spectral fragmentations (Figure 1) leads to revealing insights into the structures considered

The preliminary facile decomplexation resulting in the $MH^+-Cr(CO)_2L$ fragment was followed in each case by the cleavage of the glycosidic bonds The pseudodisaccharide derived ions were observed as protonated species (3H or 4H), whereas the monosaccharide ions were recognized as oxonium type ion [9] That the labelling had taken place in the ring X or Z of kanamycin A (i e at 6'-N or 3''-N position) might be deduced from the fragmentations observed for the compounds **3aA** and **3aB** Because of the identical mass for the rings X and Z it was unfortunately not possible to distinguish between them by mass spectroscopy As far as tobramycin was concerned the glycosidic fragments observed for its derivatives **3bA** and **3bB** indicated precisely the glycosidic ring X as a site of attack of organometallic label However, two positions were still concerned, i e 6'-N or 2'-N.



Starting from kanamycin A R' = R'' = OH

Comp	L	MH^+	MH^+-$Cr(CO)_2L$	$(X+Y-1+Bz+3H)^+$ or $(Z+Y-1+Bz+3H)^+$	$(X+Y+3H)^+$ or $(Z+Y+3H)^+$	$(X-1+Bz)^+$ or $(Z-1+Bz)^+$
3aA	CO	725	589	429	325	266
3aB	$P(OMe)_3$	821				

Starting from tobramycin R' = H, R'' = NH_2

Comp	L	MH^+	MH^+-$Cr(CO)_2L$	$(X+Y-1+Bz+4H)^+$	$(X+Y+3H)^+$	$(X-1+Bz)^+$
3bA	CO	708	572	413	325	249
3bB	$P(OMe)_3$	804				

Figure 1 Principal ions and corresponding fragmentations in the FAB mass spectra of metallohaptens **3**
Bz benzoyl

^{13}C NMR has been used extensively for aminoglycoside antibiotics [8,10] The chemical shifts have been demonstrated to be practically invariant for a structurally similar series of compounds, provided there is no structural modification within three bonds of the carbon under examination Furthermore, conditions particularly the pH environment has an evident effect on the ^{13}C chemical shifts A large shielding (≈ 3-5 ppm) must be especially noted for carbon nuclei β to the protonated amino group [11] This so called "β-shift" effect coupled with the structural effects and pH-induced anomeric shifts[12] constitutes a diagnostically valuable tool , it makes assignments possible by comparison with the chemical shifts of analogous ^{13}C nuclei in the model, not substituted compounds

Carbon	3aA			3aB			3bA			3bB		
	pD11(δ_K)	pD3(δ_K)	Δ(ΔK)	pD11	pD3	Δ	pD11(δ_T)	pD3(δ_T)	Δ(ΔT)	pD11	pD3	Δ
1	51.4(0.1)	50.7(-0.3)	0.7(0.3)	51.7	51.3	0.4	50.2(-0.3)	50.0(0.3)	0.2(0.7)	50.2	50.0	0.2
2	36.6(0)	28.7(0.1)	7.9(8.0)	36.1	28.1	8.0	35.3(-0.6)	28.0(0.2)	7.3(8.1)	35.5	28.0	7.5
3	50.5(0.4)	49.5(0.6)	1.0(1.2)	49.7	48.8	0.9	49.1(-0.6)	48.9(0.3)	0.2(1.1)	48.8	49.0	-0.2
4	88.7*(0.2)	80.4(1.5)	8.3(9.6)	87.4	78.7	8.7	87.1(0.2)	78.9(1.7)	8.2(9.7)	87.7	79.1	8.6
5	75.3(0.4)	73.9*(0.1)	1.4(1.1)	74.8	73.4*	1.4	74.5(-0.2)	74.0*(-0.2)	0.5(0.5)	75.1	74.1	-0.2
6	88.8*(-0.4)	84.4(-0.5)	4.4(4.3)	89.8	85.4	4.4	88.3(-0.1)	83.6(0)	4.7(4.8)	87.4	83.8	3.6
1'	101.4(-0.1)	97.2(0.3)	4.2(4.6)	102.4	97.9	4.5	99.9(0)	94.4(0.4)	5.5(5.9)	99.9*	94.9	5.0
2'	72.4(-0.7)	72.5(0.6)	-0.1(1.2)	72.7	72.8	-0.1	49.4(0)	48.2(0.3)	1.2(1.4)	49.4	48.3	1.1
3'	73.8(-0.8)	73.1(-0.1)	0(1.4)	73.9	73.7	0.2	34.3(-0.9)	29.3(0)	5.0(5.9)	33.4	29.1	4.3
4'	72.1(-0.2)	72.1(0)	0(0.2)	72.2	72.1	0.1	66.6(0.1)	64.1(-0.5)	2.5(1.8)	66.1	64.0	2.1
5'	68.9(-4.1)	71.3(1.9)	**-2.4(3.9)**	68.2	70.3	**-2.1**	72.9*(-1.3)	74.4*(4.0)	**-1.7(3.8)**	73.2	74.7	**-1.5**
6'	41.7(-1.1)	40.7(-0.9)	1.0(1.2)	41.5	40.7	0.8	41.3(-0.6)	39.6(-1.4)	0.7(0.9)	41.0	39.7	1.3
1''	100.9(0.4)	100.5(0)	0.4(-1.0)	98.8	98.9	-0.1	100.4(0.4)	100.6(0)	-0.2(-0.5)	100.0*	100.7	-0.7
2''	73.0(-0.4)	69.1(-0.2)	3.9(4.1)	73.1*	69.1	4.0	72.3(0.1)	68.3(0.2)	4.0(4.1)	72.1	68.4	3.7
3''	55.4(-0.2)	56.0(-0.1)	-0.6(-0.5)	55.5	56.2	-0.7	55.0(0.4)	55.3(0.3)	-0.3(-0.4)	55.5	55.4	0.1
4''	70.5(-0.1)	66.5(-0.1)	4.0(4.0)	70.6	66.5	4.1	70.1(0.3)	65.7(0.2)	4.4(4.3)	70.4	65.8	4.6
5''	73.3(-0.1)	73.8*(0.0)	0.2(-0.4)	73.3*	73.3*	0	72.7*(0.2)	73.1(0.2)	-0.4(-0.4)	72.5	73.0	-0.5
6''	61.5(-0.1)	61.0(0)	0.5(0.6)	61.5	60.8	0.7	61.0(0.2)	60.2(0.2)	0.8(0.8)	61.1	60.4	0.7
C=O	168.6	168.4		169.0	170.5		167.4	167.8		167.5	169.6	
C=O	198.2	198.2		181.9	187.3		197.2	197.0		181.1	186.6	
C-BCT	93.6, 94.8	93.2, 95.2		89.8, 90.2	90.0, 90.1		95.0, 96.2	95.0, 97.3		91.4, 92.4	91.5, 92.3	
	97.4, 97.7	97.7, 98.9		90.6, 93.7	90.8, 94.5		98.7, 99.6	98.6, 99.3		92.6, 94.9	92.7, 95.7	
P-OMe				50.1	50.1					50.8	50.9	

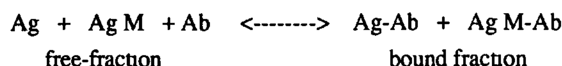
Table 2. ^{13}C-chemical shifts of compounds 3 together with deuteronation shifts Δ (pD11-pD3)
(δ_K) = δ(3aA)-δ(kanamycin A) ,[10c] (δ_T) = δ(3bA)-δ(tobramycin)[8a]
ΔK and ΔT : deuteronation shifts for kanamycin A[10c] and tobramycin[8a]
* Arbitrary assignments, may be interchanged within any column

The exact sites of metallolabelling of **1a** and **1b** were deduced in this way from the pH-dependent ^{13}C NMR study (Table 2) A change in pD from 11 to 3 caused the ≈ 4 ppm upfield shift of C4'' and C2'' in **3aA** and **3aB**, similar to that observed for the parent **1a** , such deuteronation shifts were indicative of the presence of the free C3''-amino groups in these metallohaptens Similarly, the C3' and C1' resonances of **3bA** and **3bB** exhibited the β-shift effects, which indicated the presence of the amino groups in their C2' positions Following these statements, one can eliminate 3''-N position of **1a** and 2'-N position of **1b** among the potential sites of labelling, as advanced above by mass spectroscopy The only remaining 6'-N position was in fact the exact site of labelling both in kanamycin A (**1a**) as well as in tobramycin (**1b**) Thus in contrast to the parent **1a** and **1b**, the C5' (β) resonances in **3aA, aB, bA** and **bB** underwent no acid upfield shift The large lowering of the corresponding Δ values (Δ = -1 5 to -2 4 compared to Δ ≈ +4 for **1a** and **1b**) was also due to the shielding of C5', particularly pronounced in the non-acidic medium Such an upfield shift was typically caused by the N-acylation of an amino group in the β-position

The differences in basicities of the kanamycin A and the tobramycin amino groups[6,12c] appeared to be sufficient to allow us to prepare the mono metallolabelled (at 6'-N position) derivatives with a good selectivity Owing to the permanent presence of one H_2N-CH_2 fragment in other compounds of the series, we consider the presented approach as generally useful for the selective metallolabelling of aminoglycoside antibiotics

Immunoassay technique

Due to their ability to quantitate quite specifically a great number of analytes the immunoassay methods have become increasingly important in the field of biomedical analysis Here we propose the metalloimmunoassay of aminoglycoside drugs in a competitive approach Immunoassays are largely based upon the competitive binding that occurs between a labelled (Ag M) and unlabelled ligand (Ag) for highly specific receptor sites on antibodies (Ab) The scheme of this assay is outlined as follows



Fixed amounts of both Ag M and Ab are mixed with increasing amounts of free analyte (Ag) to obtain a calibration curve which provides the means of determining the quantity of the analyzed substance in unknown samples After a determined incubation time, the detection of the label is performed by measuring physical or chemical properties associated to the label Separation of the free labelled analyte from that of the label present in the bound fraction could be accomplished most immunoassay models So, a standard curve is able to be constructed In the competitive metalloimmunoassay technique described here the metal contained in the organometallic label could be performed by different analytical methods as recently described for other metallohaptens [14-15] Atomic absorption spectrometry was chosen in this work to detect the chromium present into the label because this technique has become sensitive and suitable to quantifying samples in the form of microvolumes [16]

Before determining inhibition of the metallolabelled drug we found that the organochromium-labelled aminoglycosides were detected in the concentration range from 50 to 2000 ng ml^{-1} after introduction of 25 µl aliquots into the graphite furnace of the atomic absorption spectrometer This technique provides a good range for the detection of this category of antibiotics which appear in the serum at the level higher than 1 µg ml^{-1} [17]

Using commercial antibodies from tobramycin typical standard curves could not be obtained, probably because the concentration of the antibody samples was too low In order to improve this assay we have been starting the production of polyclonal antibodies in our laboratory, both against tobramycin and kanamycin A The antisera obtained will be tested under the form of titration curves Moreover, the study of cross-reactivity, and the assessment of the recognition capability of the tracer for the antibodies must be evaluated If the immunological reagents have satisfactory specificity, a standard curve will be recorded and clinical samples tested The results abtained will be correlated with those found in a commercial techniques Immunological investigations are in progress and will be published in the future

EXPERIMENTAL SECTION

General All operations were carried out under argon using vaccum line techniques Kanamycin A (monosulfate) and tobramycin (free base) were purchased from SIGMA-Chimie S A R L Metallocenic "activated" ester **2A** was prepared according to published procedure [5] Mass spectra were obtained by positive ion FAB MS technique employing thioglycerol as the matrix solvent ^{1}H and ^{13}C NMR spectra were recorded at 400 and 100 53 MHz, respectively Proton decoupled ^{13}C NMR spectra were obtained at 25° C in D_2O with dioxane as an internal standard The use of a spectral width of 25 kHz with a memory of 32K gave a digital resolution of 1 5 Hz The pH of each sample was adjusted with dilute NaOD and DCl for the benchrotrenic derivatives **3aA** and **3bA** or Robinson buffer (0 04 M/L CH_3CO_2H, 0 04 M/L H_3PO_4, 0 04 M/L H_3BO_3) for the phosphite groupe containing derivatives **3aB**, **3bB**

N-Succinimidyl Benzoate Chromium Dicarbonyl Trimethylphosphite (2B) This ester was prepared according to the procedure reported previously[5], starting from the corresponding carboxylic acid[13] and N-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide Crystallization of the crude product from toluene/hexane afforded **2B** (yield 60%) as orange crystals m p 84° C , IR (KBr) ν 1931, 1860, 1765, 1746 , ^{1}H NMR (CD_3COCD_3) δ 5 89 (d , J = 6 3 Hz, 2H) 5 15 (dt, J = 6 3 Hz, J = 2 4 Hz, 2H) 5 60-5 53(m,1H), 3 53(d, J_{PH} = 11 2 Hz, 9H), 2 89(s,4H) , ^{13}C {^{1}H} NMR (CD_3COCD_3) δ 236 3, 170 7, 164 5, 93 9, 92 8, 87 0, 79 8, 52 0, 26 7 , MS m/z 451(M^+,79), 420(15), 337(36) Anal Calcd for $C_{16}H_{18}NO_9PCr$ C, 42 57 , H, 3 99 Found C, 42 96 , H, 4 17

General Procedure for the Synthesis of Metallohaptens 3 In the reactions employing kanamycin A monosulfate, the antibiotic-free base was liberated at first by adding $NaHCO_3$ (2 molar excess) to the aqueous solution of the drug In a typical reaction a solution of the organometallic label (0 4 mmol) in 20 mL of THF was added via syringe to a stirred solution of the drug (0 4 mmol) in 10 mL of water, at room temperature The mixture was stirred in the dark under argon over 4 to 6h period After removal of the solvent in vacuo, the solid was washed with THF in order to remain traces of the unreacted label Purification by flash chromatography (silica gel 230-400 mesh, eluent $CHCl_3/MeOH/NH_4OH$ = 6/8/3, under argon) afforded 3 as yellow (**3aA** and **3bA**) or orange (**3aB** and **3bB**) platelets Yield **3aA**-89 %, **3bA**-81 %, **3aB**-47 %, **3bB**-45 % Metallohaptens 3 decomposed at 200-220° C Their spectral data (IR, NMR, MS) are reported in the text

Immunoassay Procedure. Assays were performed in 12x75 mm glass tubes containing 50 µl of metallohapten conveniently diluted in phosphate buffer saline 0 01M pH 7 2 (noted PBS), 50 µl of 1/200 dilution in PBS of anti-tobramycin antiserum, 50 µl of various quantities of tobramycin diluted in PBS and 350 µl of PBS The mixture was gently wortexed 15s and incubation was performed 2h at room

temperature Separation of the free and bound fractions was obtained by selective solvent extraction as previously reported [14] In this experiment 500 µl of ethyl acetate was added to each tube, the mixture was gently vortexed 10s, and after standing for 10min, 25 µl of the organic layer containing free metallohapten was introduced into the graphite furnace of the atomic absorption spectrometer All samples were assayed in duplicate and each tube in triplicate and appropriate controls were included in the assay

Apparatus. Atomic absorption measurements were carried out on a Hitachi model Z 7000, equipped with a Zeeman corrector system Instrument settings were chromium hallow cathode 7 5 mA, wavelenght 359.3 nm, spectral bandwidth · 0.2 nm, non pyrolytical graphite tubes were used and 25 µl of the sample introduced into the tubes Optimal conditions for atomization were . dry at 120°C for 10s (ramp time 20s); char at 1000°C for 10s (ramp time 30s), atomization at 2900°C for 5s (ramp time 0s) During the atomization, the internal argon gas flow was stopped

Antibodies Polyclonal antibodies against tobramycin were obtained from Sigma-chimie S A R L

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16 Nevertheless, the infrared FT spectroscopy would be a technique of choice if the simultaneous detection of both metallotracers are needed The introduction of the phosphite ligand in **3aB** and **3bB** makes it possible to differentiate the carbonyl frequencies in this case

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