



Complete structure analysis of OR-1746, a complex product of cyclocondensation of arylhydrazomalononitriles containing clusters of protonated and unprotonated nitrogens, by pulsed-field-gradient heteronuclear NMR

Piero Pollesello*, Pentti Nore

Orion Pharma, Cardiovascular Research and Development, P.O. Box 65, FIN-02101 Espoo, Finland

Received 20 August 2002; received in revised form 8 October 2002; accepted 8 October 2002

Abstract

OR-1746, or {4-Ethoxy-6-imino-5-[4-(4-methyl-6-oxo-1,4,5,6-tetrahydropyridazin-3-yl)phenyl]hydrazono}-5,6-dihydro-1*H*-pyrimidin-2-ylidene}-[4-(4-methyl-6-oxo-1,4,5,6-tetrahydropyridazin-3-yl)phenylazo]-acetonitrile is the product of the cyclocondensation of two molecules of the arylhydrazomalononitrile levosimendan (CAS registry number [141505-33-1]) with ethanol. OR-1746 is a molecule with a complex structure containing clusters of protonated and unprotonated nitrogens. Its structure was only partially elucidated by elemental analysis and by conventional NMR. However, the presence of many unprotonated nitrogen atoms did not allow the unambiguous assignment of the ^1H , ^{13}C and ^{15}N NMR spectra with short-range heterocorrelated techniques, or even with traditional long-range 2D experiments. Pulsed-field-gradient heteronuclear multiple bond coherence sequences (PFG ^1H - ^{13}C and PFG ^1H - ^{15}N) were, therefore, used to fully assign the NMR spectra and elucidate the chemical structure of OR-1746. By using these techniques, long-range couplings between protons and carbons or proton and nitrogen atoms as distant as five bonds in the structure were detected without losing the signals of the protonated heteroatoms. The long range coupling information provided by the novel NMR experiment can be used effectively in the complete structure determination of complex molecules containing clusters of protonated and unprotonated nitrogens.

© 2002 Elsevier Science B.V. All rights reserved.

Keywords: OR-1746; Levosimendan; Pulsed-field-gradient NMR; Arylhydrazomalononitriles; Cyclocondensation

1. Introduction

The compound OR-1746, or {4-Ethoxy-6-imino-5-[4-(4-methyl-6-oxo-1,4,5,6-tetrahydropyridazin-3-yl)phenyl]hydrazono}-5,6-dihydro-1*H*-pyrimidin-2-ylidene}-[4-(4-methyl-6-oxo-1,4,5,6-tetrahydropyridazin-3-yl)phenylazo]-acetonitrile (Fig. 1, I) belongs to a family of compounds developed

* Corresponding author. Tel.: +358-504-29-4191; fax: +358-104-29-2924.

E-mail address: piero.pollesello@orionpharma.com (P. Pollesello).

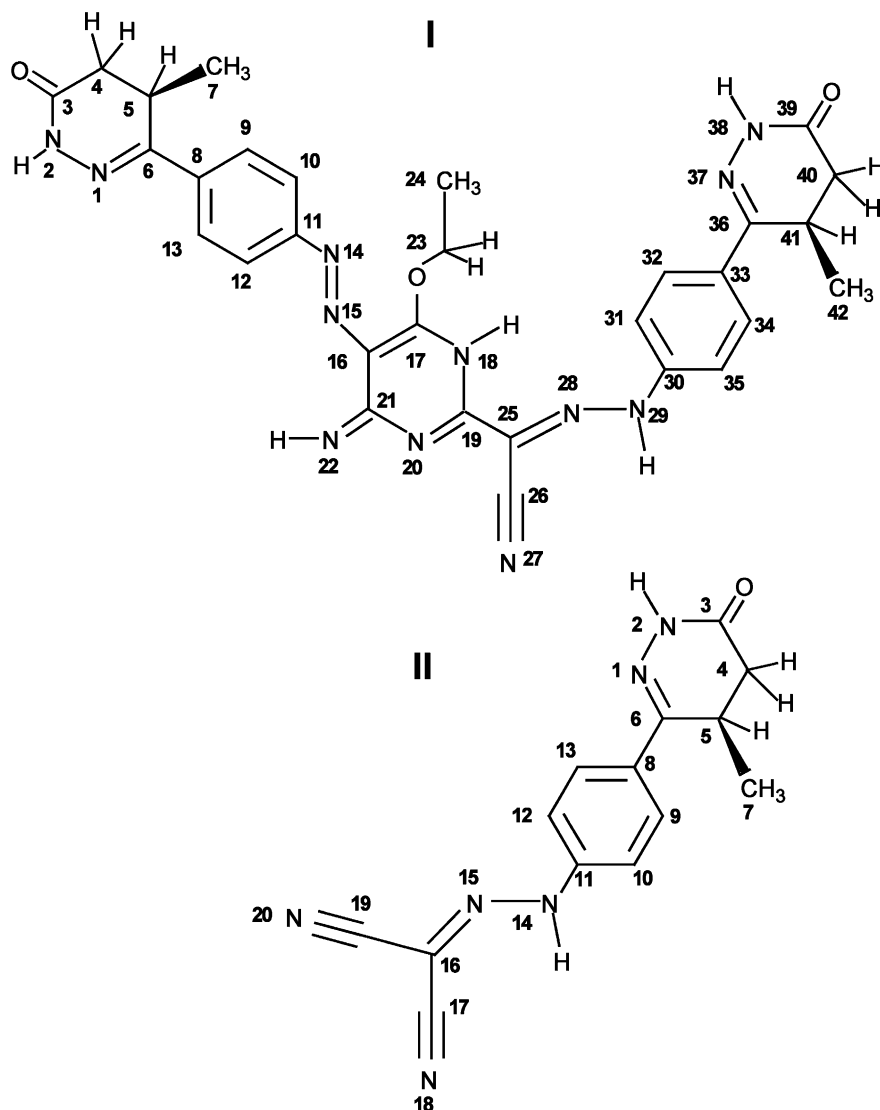


Fig. 1. **(I)** The compound OR-1746, {4-Ethoxy-6-imino-5-[[4-(4-methyl-6-oxo-1,4,5,6-tetrahydropyridazin-3-yl)phenyl]hydrazono}-5,6-dihydro-1*H*-pyrimidin-2-ylidene}-[4-(4-methyl-6-oxo-1,4,5,6-tetrahydropyridazin-3-yl)phenylazo]-acetonitrile; **(II)**: levosimendan, CAS registry number [141505-33-1].

for the treatment of congestive heart failure in patients with acute myocardial infarction. The lead compound, levosimendan (Fig. 1, II)[1], induces a positive inotropic effect [2] by increasing the calcium sensitivity of myocardial contractile proteins by binding to a specific site on human cardiac troponin C [3]. OR-1746 has a structure typical of the products of the cyclocondensation of

arylhydrazomalnonitriles with ethanol: the compound is in fact obtained by making the cyano groups of the malononitrile of two levosimendan molecules react with an ethanol molecule according to Schaefer et al. [4].

HPLC connected to diode array UV-detector and elemental analysis were used, respectively, to check the purity and to characterize partially the

product. To solve the structure, 1D NMR spectra were obtained, which showed well resolved peaks belonging to the two levosimendan moieties and to the etoxy residue. The use of short-range hetero-correlated techniques and traditional long range ^1H - ^{13}C 2D experiments, however, did not allow a full and unambiguous assignment of the 1D spectra due to the presence of many unprotonated carbon and nitrogen atoms in the central part of this cyclocondensation product.

It has been shown that by using pulsed field gradients to select specific coherences [5,6], it is possible to replace phase-cycling in many multiple-pulse experiments. The main advantages, which result from gradient selection of coherence, are the elimination of subtraction error and an impressive reduction in the total experimental time. Recently, F_1 -selective gradient-enhanced HMQC and HSQC methods have been used to observe chemical-shift correlations between directly bound protons and heteronuclei [7]. Pulsed-field-gradient enhanced heteronuclear multiple bond coherence NMR sequences have been proposed also as tools to enhance the detectability of small couplings [8].

By using these techniques, long-range couplings between protons and carbons (^1H - ^{13}C) or protons and nitrogen atoms (^1H - ^{15}N) as distant as five bonds in the structure of OR-1746 were detected. The evidence of such very long-range couplings gave an invaluable structural information that allowed the unambiguous determination of the structure of OR-1746.

2. Materials and methods

2.1. Chemistry

OR-1746 was produced at the Department of Synthetic Chemistry of Orion Pharma starting from levosimendan by a reaction described by Schaefer et al. [4] for the cyclocondensation of arylhydrazomalononitriles with ethanol. Briefly, a mixture of levosimendan (60 g) and triethylamine (21 ml) in ethanol (4000 ml) was stirred over night at 50 °C. The hot mixture was filtered. The residue was triturated in the presence of acetonitrile (250 ml) and dried in vacuo at 40 °C. The

yield of the resulting brown-red product (with melting point 219–221 °C) was 17.9 g.

2.2. HPLC and elemental analysis

The purity of OR-1746 was determined by HPLC using a Hewlett-Packard HPLC system model 1090 with photodiode array detector connected to the HP Vectra VL2 4/66 MHz workstation. A Novapak C-18 100 RP-18 column (3.9×125 mm, 4 μm) was used. As mobile phase, a gradient of (A) phosphate buffer pH 2.1 and (B) methanol (20–80% of B in 0–20 min) was used with a flow rate of 1.0 ml/min. The wavelengths of the UV-detector were 300 and 380 nm. The purity of OR-1746 was 96.8% in the batch which was used for further analyses.

It was not possible to grow suitable single crystals for an X-ray structural analysis. The exact mass measurement of OR-1746 was carried out with Q-ToF 2 mass spectrometer (Micromass Ltd., Manchester, UK) using positive electrospray as an ionisation mode. The deviation of the protonated molecule ($\text{C}_{30}\text{H}_{31}\text{N}_{12}\text{O}_3$; 607.2637) from the theoretical value was -0.8 ppm (i.e. -0.5 mmass).

2.3. NMR spectroscopy

Three hundred milligrams of OR-1746 were dissolved in 600 μl DMSO for NMR analysis. NMR experiments were performed at 27 °C on a Bruker ARX 400 NMR spectrometer operating at a proton frequency of 400.13 MHz, equipped with an inverse detection broadband probe and pulsed field gradient coils, using 5 mm (o.d.) sample tubes. Chemical shifts were referred to tetramethylsilane (TMS).

^1H spectra were acquired using a 60 ° pulse, recycling time of 4 s, spectral width of 5600 Hz, and data size of 32 k points. ^{13}C and ^{15}N decoupled direct detected spectra were acquired using 32 k data points and WALZ-16 decoupling during acquisition (and for ^{15}N also during relaxation) with a repetition time of 2 s. The ^{15}N spectra were referenced against the spectrum of enriched formamide which gives a peak at 270 ppm.

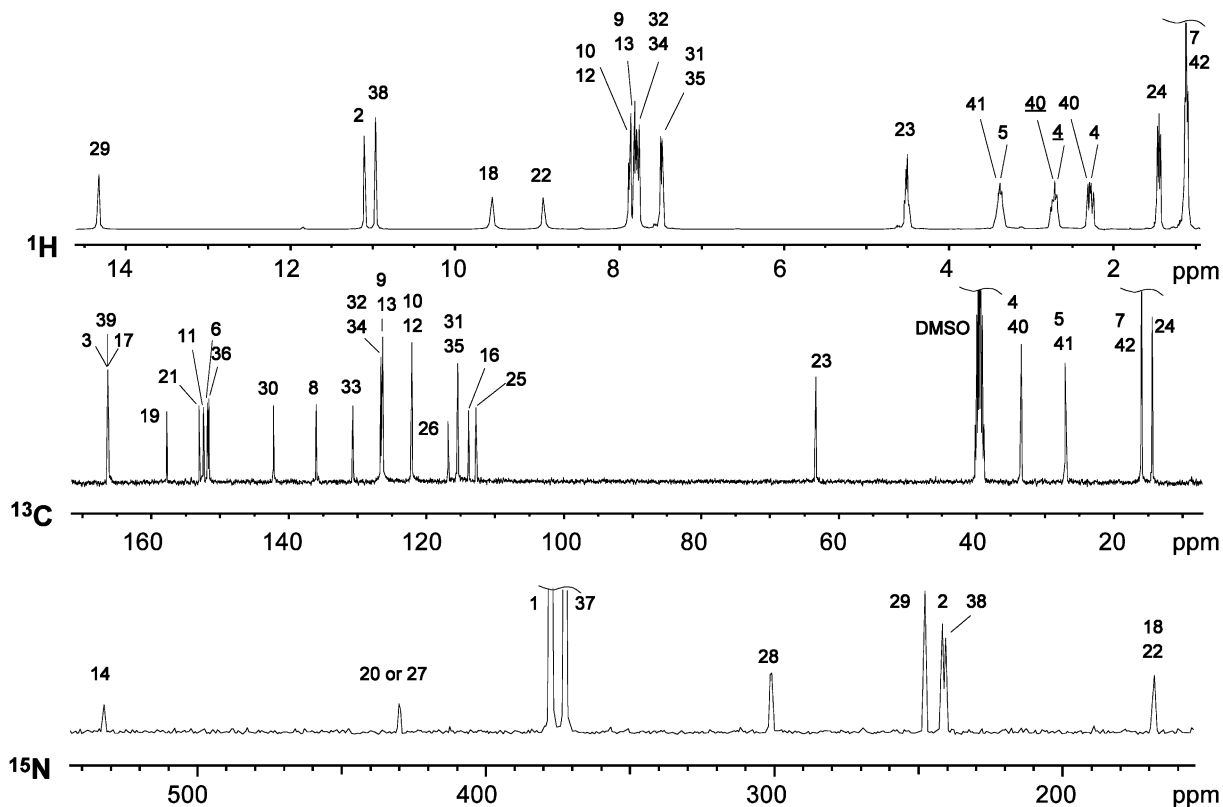


Fig. 2. 1D NMR spectra of OR-1746 in DMSO. From top to bottom: ^1H -, ^{13}C - and ^{15}N -NMR spectra. The assignments are indicated by numbers referring to the structures I and II (see Fig. 1).

2.4. ^1H - ^{13}C PFG-HMBC

Proton detected pulsed-field-gradient heteronuclear multiple-bond ^1H - ^{13}C correlation spectroscopy (PFG-HMBC) were recorded using the simple pulse sequence with z-gradients proposed by Hurd and Jones [9]. Briefly, this sequence generates multiple quantum coherence for protons directly bound to ^{13}C nuclei. Thereafter, two symmetric gradients about a proton π pulse leave zero net phase for all protons. Finally, by means of a third gradient applied just before acquisition, the magnetisation of protons coupled to ^{13}C can be selectively refocussed.

The amplitudes of the three sine-bell shaped gradients, calculated from the gyromagnetic ratios of ^1H and ^{13}C , were 9:9:4.71 G/cm, respectively. The gradient pulses were of 2 ms, the delay for the

gradient recovery was 100 μs and the relaxation delay 1.5 s. The delay ($1/2J_{\text{CH}}$) for the evolution of the long range coupling was selected in the range from 60 to 300 ms in order to maximise the information on different coupling in the same spectrum. We noticed that the spectrum obtained using a delay of 300 ms gave informations on connectivities distant as long as five-bonds without loosing the signals of the protonated heteroatoms.

The spectra were acquired over an F_2 spectral width of 5600 Hz and an F_1 width of 17 500 Hz. The number of transients was 32–128 per increment and 512 increments were collected.

In both cases, the data were zero-filled to give $2 \times 1 \times 1$ k data matrices and processed with sine-bell functions in both domains. Data were expressed in magnitude mode after Fourier transformation.

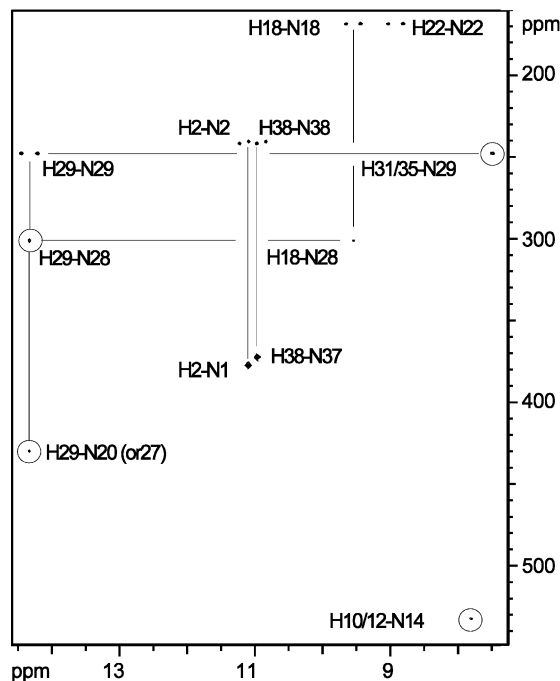


Fig. 3. Pulsed-field-gradient proton detected heteronuclear ^1H - ^{15}N correlation spectrum (PFG-HMBC) of OR-1746. Expansion from 160 to 550 ppm in F_1 and from 7.2 to 14.2 ppm in F_2 . The spectrum, obtained by using a delay of 100 ms for the evolution of the long range couplings, gives informations on proton-nitrogen connectivities distant as long as five-bonds without losing the signals of the protonated nitrogens. Cross peaks of all the nitrogens directly connected to a proton are doublets because no decoupling during acquisition was used. The cross peak marked as H29-N20 could, in theory, be due to a coupling H29-N27. However, H29 does not show in the spectrum ^1H - ^{13}C PFG-HMBC any coupling with C26 but with C19. Thus we assigned the signal at 430 ppm to N20.

2.4.1. ^1H - ^{15}N PFG-HMBC

In the case of ^1H - ^{15}N PFG-HMBC experiments the three sine-bell shaped gradients had values of 9:9:1.8 G/cm, respectively. The gradient pulses, the delay for the recovery of the gradient and the relaxation delay were as described for the ^{13}C - ^1H experiments. The delay for the evolution of the long range coupling was selected in the range from 20 to 100 ms. The spectra were acquired without decoupling. The number of transients was 32–128 per increment and 512 increments were collected.

3. Results and discussion

The 1D proton, carbon and nitrogen NMR spectra of OR-1746 are shown in Fig. 2. The unambiguous assignment of all proton, carbon and nitrogen resonances was made possible by analysing the ^1H - ^{13}C and ^1H - ^{15}N 2D NMR spectra as follows.

We started by the assignment of all the nitrogens directly bound to protons, which is straightforward because those nitrogens give doublet signals in the non-decoupled PFG-HMBC. Namely, N29 at 220 ppm, N2 at 214 ppm, N38 at 215 ppm, N18 and N22 at 180 ppm are assigned due to their stronger coupling with H (Fig. 3).

The assignment was continued by the identification of the N29-H29 cross peaks centered at δH 16 ppm in the ^1H - ^{15}N non-decoupled spectrum. From the same proton, two long range couplings to nitrogen atoms can be seen: a stronger one to N28 and a weaker one to N20. A coupling between N28 and a proton tentatively assign as H18 can be seen. The latest assignment was confirmed in the ^1H - ^{13}C spectrum (Fig. 4), in which it can be seen that C16 is coupled both with H22 and H18.

Furthermore, in the ^1H - ^{13}C spectrum it can be seen that H29 is coupled on one side to C31, C35 and C30 and on the other side to C25 and C19, and that C17 is coupled to H23 on one side and H22 on the other, thus confirming the covalent bond with the ethoxy residue.

All the aromatic protons and carbons can be assigned (see inset of Fig. 4): in fact, the all the protons H9, H13, H10 and H12 of the first aromatic ring are connected to C11 and, similarly, the protons H31, H32, H34, and H35 are coupled with C30, which in turn is also coupled with H29.

The protons of two pyridazine rings are well resolved and all the protons and carbons can be assigned: of use is the coupling of C6 both with H2, H4, H5, and H7 as well as with H9 and H13. In the same way C36 is connected to H38, H40, H41, and H42 as well as with H32,-34.

It is known that the stereochemistry of the two levosimendan moiety does not change upon the formation of the cyclocondensation product. The difference in the coupling of C6 with H4 and H4 and of C36 with H40 and H40 is consistent with

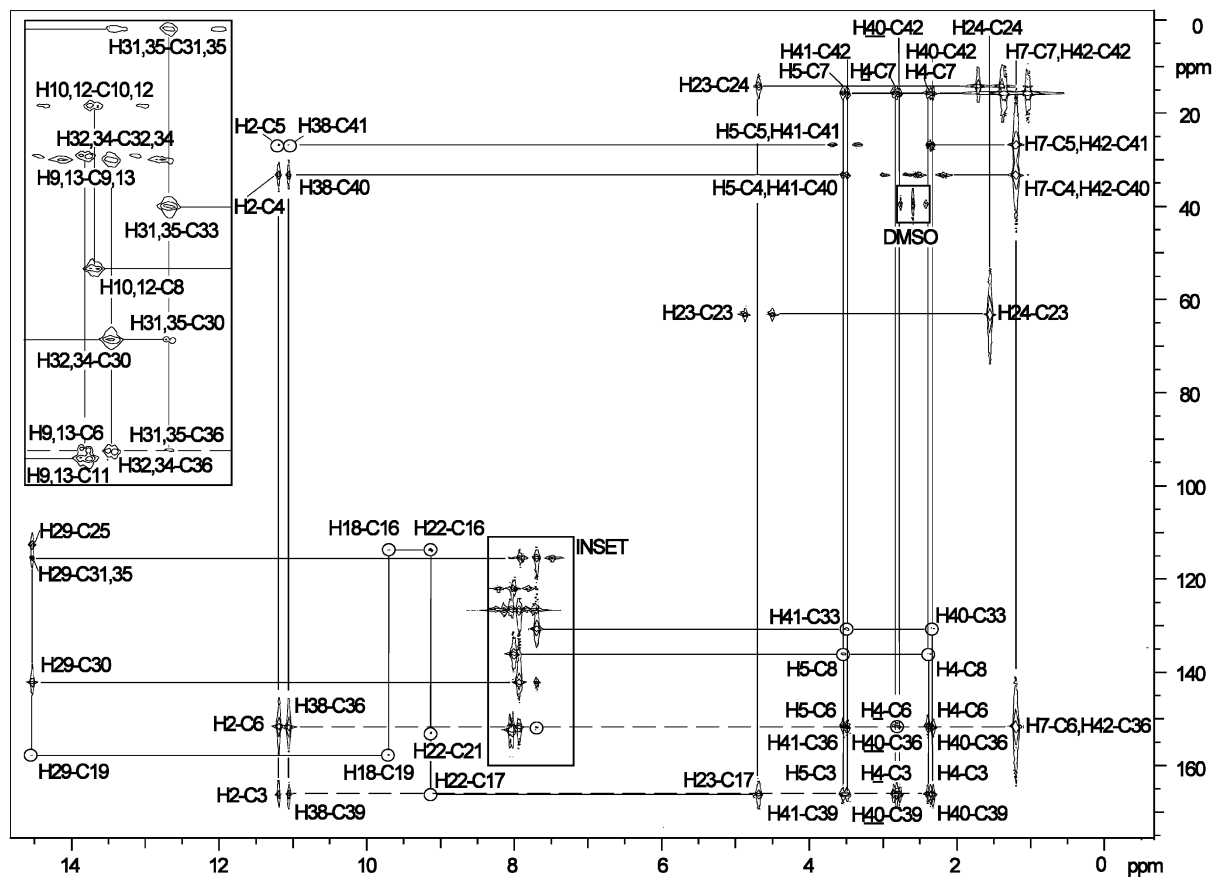


Fig. 4. Pulsed-field-gradient proton detected heteronuclear ^1H - ^{13}C correlation spectrum (PFG-HMBC) of OR-1746. Expansion from -3 to 176 ppm in F_1 and from -0.7 to 14.7 ppm in F_2 . The spectrum, obtained by using a delay of 300 ms for the evolution of the long range couplings, gives informations on proton-carbon connectivities distant as long as five-bonds without losing the signals of the protonated carbons. Cross peaks of carbons directly connected to a proton are doublets because no carbon decoupling during acquisition was used.

the (+)-enantiomer since the antiperiplanar protons (H4 and H40 has dihedral angle $+165 \pm 15^\circ$ with H5 and H41, respectively) has a smaller coupling with C6 and C36 than the synclinal (H4 and H40, with dihedral angle $-60 \pm 30^\circ$ with H5 and H41, respectively).

To complete the assignment, it can be seen from the ^1H - ^{15}N spectrum that H2 and H38 generate cross peaks with N1 (375 ppm) and N37 (378 ppm), respectively. Moreover, H10 and H12 show significant correlation to N14 at 530 ppm.

By coupling the data of the ^1H - ^{13}C and ^1H - ^{15}N spectra, it is possible to track all the protons, carbons and nitrogen of the molecule from end to

end, assigning unambiguously the NMR signals on the 1D spectra. Only C26 (on the cyan group) and N15 did not show coupling with any proton and were assigned per exclusion. The assignments are given in Table 1. In the same table, for comparison, the ^1H -, ^{13}C - and ^{15}N -NMR spectral data of levosimendan are given.

In conclusion, the simple gradient assisted HMBC pulse sequences used for the 2D heteronuclear spectra, originally proposed by Hurd and John (9), gave informations on connectivities distant as long as five-bonds without losing the signals of the protonated heteroatoms. This rapid technique allows the unambiguous assignment and

Table 1

Assignment of the ^1H -, ^{13}C - and ^{15}N -NMR signals of levosimendan and OR-1746

Levosimendan			OR-1746								
Atom	δ 1H	δ 13C	δ 15N	Atom	δ 1H	δ 13C	δ 15N	Atom	δ 1H	δ 13C	δ 15N
1			375	1			378	37			372
2	11		240	2	11.1		242	38	11		241
3		166		3		166.1		39		166.1	
4 ^a	2.7	33.3		4 ^a	2.69	33.4		40 ^a	2.66	33.4	
4 ^b	2.3			4 ^b	2.29			40 ^b	2.26		
5	3.4	26.8		5	3.4	26.9		41	3.36	26.9	
6		151.5		6		151.6		36		151.4	
7	1.08	15.7		7	1.12	15.9		42	1.12	15.9	
8		141.7		8		135.8		33		130.5	
9, 13	7.84	126.8		9,13	7.81	126.5		32,34	7.77	126.2	
10, 12	7.52	116.3		10,12	7.88	122		31,35	7.49	115.3	
11		131.8		11		152.3		30		142	
14	13.1		500	14			532	29	14.3		248
15			n.d.	15			n.d.	28			301
16		85.1		16		113.6		25		112.6	
17 ^c		109.6		17				26			
18				18	9.55		169	27			430 ^c
19 ^c		114		21		152.8		19		157.6	
20				22	8.93		169	20			430 ^c
				23	4.5	63.2					
				24	1.45	14.3					

^a Antiperiplanar compared with proton 5/41.^b Synclinal compared with proton 5/41.^c Assignment interchangeable.

complete structural analysis of a complex organic molecule containing clusters of protonated and unprotonated nitrogens.

References

- [1] R. Bäckström, J. Haarala, E. Honkanen, P. Nore, T. Wikberg, H. Haikala (1992) Patent No. GB 2251615 A1 920715.
- [2] I. Edes, E. Kiss, Y. Kitada, F.M. Powers, J.G. Papp, E.G. Kraniasand, R.J. Solaro, *Circ. Res.* 77 (1995) 107–113.
- [3] P. Pollesello, M. Ovaska, J. Kaivola, C. Tilgmann, K. Lundström, N. Kalkkinen, I. Ulmanen, E. Nissinen, J. Taskinen, *J. Biol. Chem.* 269 (1994) 28584–28590.
- [4] H. Schaefer, K. Gewald, M. Gruner, *J. Prakt. Chem.* 331 (1989) 878–883.
- [5] A. Bax, P.G. De Jong, A.F. Mehlkopf, J. Smidt, *Chem. Phys. Lett.* 69 (1980) 567–570.
- [6] P. Barker, R. Freeman, *J. Magn. Reson.* 64 (1985) 334–338.
- [7] J.-M. Bernassau, J.-M. Nuzillard, *J. Magn. Reson. A* 108 (1994) 248–254.
- [8] P. Pollesello, O. Eriksson, E. Geimonen, F. Vittur, S. Paoletti, R. Toffanin, *NMR Biomed.* 8 (1995) 190–196.
- [9] R.E. Hurd, B.K. John, *J. Magn. Reson.* 91 (1991) 648–653.