A New Method for Distinguishing between Enantiomers and Racemates and Assignment of Enantiomeric Purity by Means of Solid-State NMR. Examples from Oxazaphosphorinanes

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Abstract: It is shown that enantiomers and racemates that have identical isotropic NMR chemical shift as well as anisotropic chemical-shift tensor parameters can be easily distinguished by means of the ODESSA (*O*ne *D*imensional *E*xchange *S*pectroscopy by *S*ideband *A*lternation) technique. The method is based on the fact that the molecular symmetries and packing of enantiomers and racemates are usually significantly different. The power of the proposed approach is demonstrated by employing as model compounds P-chiral oxazaphosphorine derivatives, which are widely used in clinical oncology. Correlation of the amplitude of the ODESSA decay (AOD) with enantiomeric excess is also presented.

Keywords: analytical methods · cyclophosphamide · enantiomeric purity · ODESSA · phosphorus · solid-state NMR spectroscopy

Introduction

The enantiomeric purity of a chiral compound is usually determined by measurement of its optical rotation or in favorable circumstances by NMR spectroscopy.[1] Liquid-phase NMR spectroscopy employing chiral solvents, liquid-crystalline organic solutions, or chiral discriminating agents (CDA) can be used to separate signals deriving from two enantiomers.[2] Chiral discrimination can be observed through a difference in the order-sensitive NMR parameters like proton–proton, proton–carbon, and carbon–carbon residual dipolar coupling, carbon chemical-shift anisotropies, and deuterium quadrupolar splittings. In such cases, the enantiomeric excess can be determined from the relative NMR signal intensities. Hill et al. revealed that ^{13}C high-resolution solid-state NMR can be considered as an interesting alternative technique.[3] This approach exploits the fact that a pure enantiomer and a true racemate provide crystals belonging to different point groups[4] and can give observable differences for the isotropic chemical shifts. As shown later on by Jakobsen and co-workers,[5] also simple integration (or deconvolution) of ^{31}P magic-angle-spinning (MAS) NMR signals with differing isotropic chemical shift can be used to determine the enantiomeric purity of organophosphorus compounds with satisfying accuracy. However, the problem arises when enantiomers and racemates show identical isotropic chemical shifts. To our knowledge, a NMR method that allows distinguishing features of the enantiomeric excess for such compounds to be visualized has not been proposed so far.

In this paper we report a new and straightforward approach that permits differences between enantiomers and racemates to be recognized and the enantiomeric excess to be assessed, by exploring differences in the internuclear distances between chemically equivalent sites (having identical isotropic chemical shifts) from the 1D NMR ODESSA (*O*ne *D*imensional *E*xchange *S*pectroscopy by *S*ideband *A*lternation) technique.[6] The ODESSA method, which uses the pulse sequence shown in Figure 1, is a rotor-synchronized, magic-angle spinning (MAS) exchange experiment, with the preparation period fixed to one-half a rotation period, $\tau_1 = \tau_R/2$,

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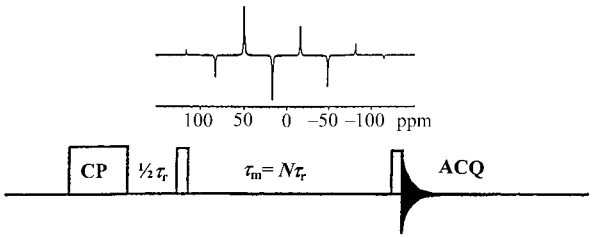


Figure 1. ODESSA pulse sequence. The preparation period is half a rotation and the mixing time is an integer number of rotation. CP = cross polarization, ACQ = acquisition.

and the mixing time equal to an integer number of rotation periods, $\tau_m = N\tau_r$.

By taking advantage of the inhomogenous character of the chemical-shift tensor anisotropy interaction, the experiment ensures that, at the beginning of the mixing time, the magnetizations associated with the various spinning sidebands are polarized in alternate directions (see top inset in Figure 1). This creates an initial difference polarization, and spin exchange results in redistribution of the polarization and a corresponding decay in the sideband intensities.

In the present work, we take advantage of the well-known fact that the molecular symmetries and packing of enantiomers and racemates are usually significantly different. We have shown previously that the ODESSA experiment is able to recognize differences in the intermolecular distances between chemically equivalent nuclei.[7, 8] Employing the preliminary observations, we assume that this experiment will also permit us to distinguish enantiomers from racemates and that it should be sensitive to different compositions of crystalline samples being present as the ground mixture or prepared in the recrystallized form. The validity of our approach is demonstrated by employing P-chiral oxazaphosphorine derivatives as model compounds.

Results and Discussion

Structural data: Cyclophosphamide (**CP**) and iphosphamide (**IF**), oxazaphosphorinane-type alkylating agents (Scheme 1), are chiral compounds widely used in clinical oncology as racemic mixtures.[9] Stec and co-workers synthesized **CP** and **IF** enantiomers[10] and found that levorotatory enantiomers are more active than racemates against several experimental tumors in mice.[11] Recently (*S*)-(−)-3-(2-bromoethyl)-*N*-(2-chloroethyl)tetrahydro-2*H*-1,3,2-oxazaphosphorine-2-amine 2-oxide ((−)**BF**) was obtained[12] and became subject of Phase I clinical trials in Poland. Intensive structural studies of **CP**, **IF**, **BF**, and their enantiomers were performed mostly by X-ray crystallography.[13–15] The only exception, which to date has not been investigated by any diffraction technique, is a racemate of a **BF** compound. This fact prompted us to carry out the X-ray studies. As we found, the racemate **BF**-rac crystallizes in a monoclinic system and the *C*2/*c* space group. Figure 2 (top) presents the molecular structure and the numbering system. The molecular packing is shown in Figure 2 (bottom). Crystal data and experimental details are shown in Table 1.

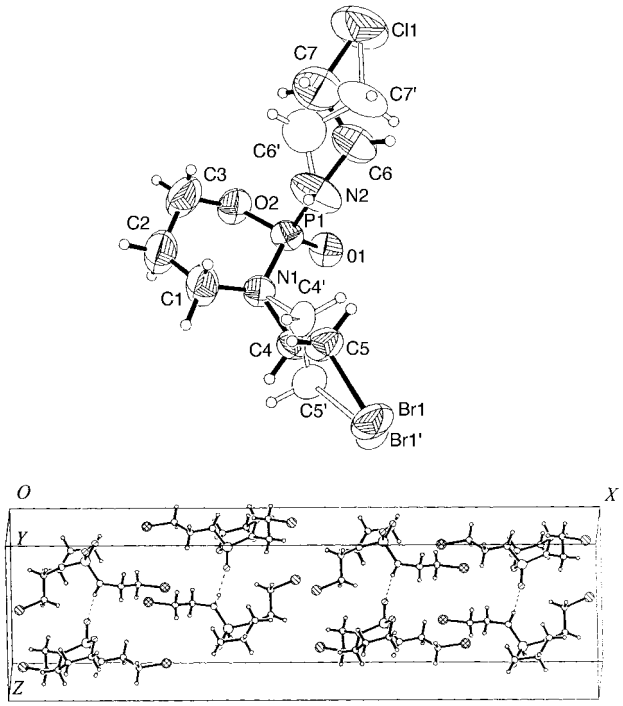
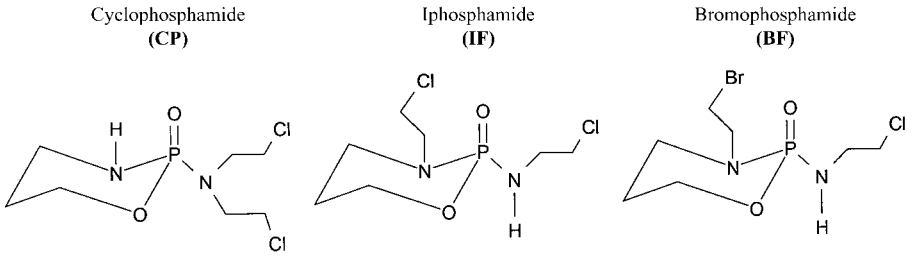


Figure 2. Top: Overall view of the **BF** racemate; the atoms with lower occupation factor are shown as open ellipsoids connected by open bonds. The ellipsoids of thermal vibration are shown with 50% probability. Bottom: Crystal packing diagram.

Solid-state NMR: Figure 3 displays ^{31}P CP/MAS (CP = cross polarization) spectra of **CP**, **IF**, and **BF** recorded at room temperature and a spinning frequency of 2.7 kHz. All spectra show a number of spinning sidebands flanking the isotropic line due to the relatively large chemical-shift anisotropy of phosphorus. The spectra of racemates and enantiomers are organized into rows. From inspection of these spectra, it is apparent that the isotropic chemical shifts and the spinning sideband patterns within a pair racemate/enantiomer are only slightly different.

This would forbid observation of separated NMR signals from racemates and enantiomers when they were present simultaneously in the same sample. In order to distinguish enantiomer from racemate in such a case, we can exploit the



Scheme 1.

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Table 1. Crystal data and experimental details for **BF**-rac.

Molecular formula	$C_7H_{15}N_2O_2PBrCl$
Formula weight	305.54
Crystallographic system	monoclinic
Space group	$C2/c$
a [Å]	35.816(6)
b [Å]	7.033(2)
c [Å]	9.956(2)
β [°]	90.42(2)
V [Å^3]	2507.6(10)
Z	8
D_c [g cm^{-3}]	1.619
μ [cm^{-1}]	7.501
Crystal dimensions [mm]	$0.15 \times 0.35 \times 0.6$
Maximum 2θ [°]	150
Radiation, λ [Å]	$Cu_{K\alpha}$, 1.54184
R_{obs} $I > 2\sigma(I)$	0.0485
R_{obs} all data	0.0576
wR_{obs} $I > 2\sigma(I)$	0.1463
wR_{obs} all data	0.1516

sensitivity of the ODESSA experiment to distance differences between chemically equivalent nuclei through proton-driven spin-diffusion rate-constant measurements.[7, 8] The rate constants W_{ij} of spin diffusion may be obtained from the intensity changes of spinning sidebands as a function of mixing time.

Figure 4 shows well how sensitive W_{ij} is to differences in intermolecular P···P distances, and how easy is to distinguish enantiomer from racemate by employing the ODESSA pulse sequence. The values of spin-diffusion rate constants and relevant r_{ij} distances taken from X-ray data are collected in Table 2.

We obtained the most dramatic difference of W_{ij}'s for **CP** samples. The **CP** racemate crystallizes in the triclinic crystal system and $P\bar{1}$ space group. In such a system, the identity or inversion (onefold rotation or rotatory-inversion axis) in any direction characterizes the symmetry. We note that the phosphorus atoms, which are involved in such a symmetry relation, are magnetically equivalent and are not recognized in the ODESSA measurements. Hence, in this case there are no magnetically nonequivalent phosphorus neighbors that would permit a spin-diffusion process. In consequence, for the **CP** racemate, excepting an initial small amplitude decay that we ascribe to the experimental imperfections, the rate W_{ij} is very slow.

Table 2 presents nearest P···P distances between magnetically nonequivalent nuclei for the five remaining systems. Having such a set of data, we were attracted by the prospect of correlation between the W_{ij} parameters and P···P distances for the nearest neighbors between magnetically nonequivalent nuclei. As can be seen in Figure 5, the ODESSA experiment indeed provides access to the visualization of different P···P distances in oxazaphosphorinanes.

Since the ODESSA experiment can so easy recognize the difference in the spectroscopic response coming from racemate and enantiomers, the obvious question was whether this approach could be also applied to test of enantiomeric excesses (*ee*). For this purpose, the racemate and (−)-cyclophosphamide were mixed in different proportions. The *ee* values were 5%, 10%, 25%, 40%, 50%, 60%, 75%, and 90%. First the ground mixture of enantiomers was examined. The results are shown in Figure 6a. As expected, the amplitude of the ODESSA decay (AOD) increases progressively with the amount of enantiomeric excess. In the next step, we recrystallized selected samples of **CP** and could see

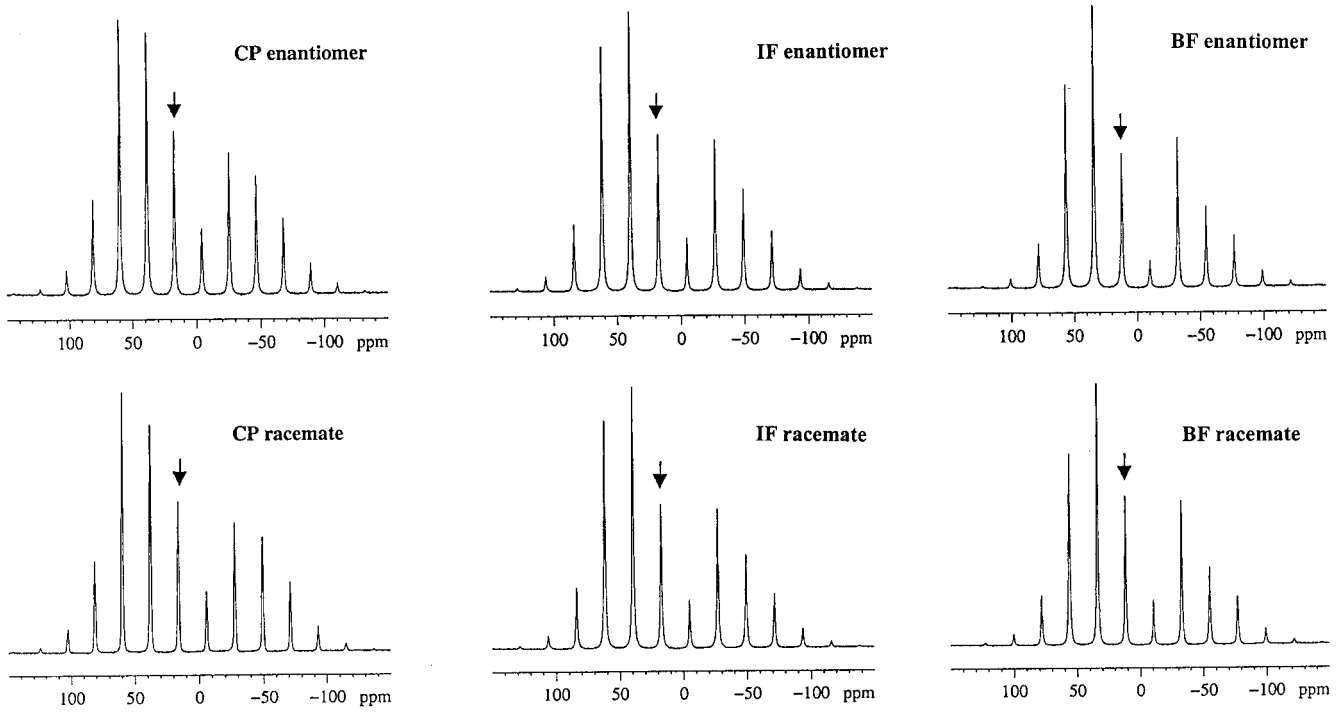

Figure 3. ^{31}P CP/MAS spectra of the oxazaphosphorinane analogues. The spectra were recorded by using a contact time of 1 ms, 120 scans and $\nu_{rot} = 2.7$ kHz. The isotropic positions are indicated by an arrow. The ^{31}P δ_{iso} values are as follow; **CP**-rac = 15.9 ppm, **CP**-en = 16.7 ppm, **IF**-rac = 12.2 ppm, **IF**-en = 11.8 ppm, **BF**-rac = 11.6 ppm, **BF**-en = 11.4 ppm.

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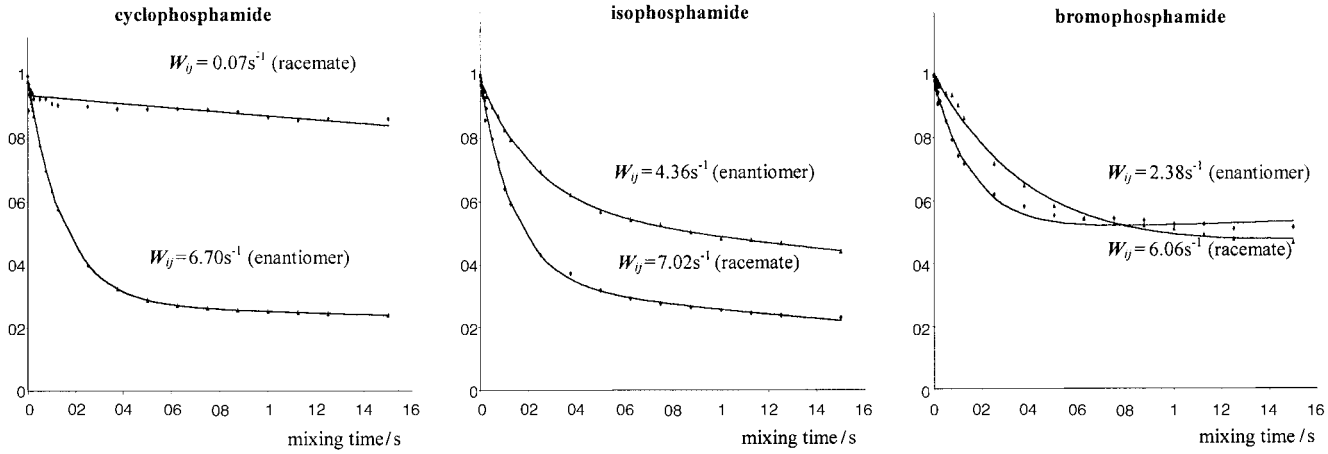

Figure 4. Intensity changes of the entire set of ^{31}P spinning sidebands for oxazaphosphorinane drugs in the ODESSA experiment as a function of mixing time.

Table 2. Values of spin-diffusion rate constants and nearest-neighbor internuclear distances (r_{ij}) from X-ray data.

	Oxazaphosphorinane	W_{ij} [s^{-1}]	P···P [Å][a]	Crystallographic system	Space group	Reference to X-ray data
1	**CP**-rac[b]	0.07	–	triclinic	$P\bar{1}$	[13a]
2	(+)**CP**[c]	6.70		rhombohedral	R_3	[13b]
3	(−)**CP**	6.70	5.042 6.255	rhombohedral	R_3	[13c]
4	**IF**-rac	7.02	4.706 6.695	orthorhombic	*Pbca*	[14a] [14b]
5	(−)**IF**	4.36	6.301 7.25	orthorhombic	$P2_12_12_1$	[14c]
6	**BF**-rac	6.06	5.05 7.95	monoclinic	*C2/c*	this work
7	(−)**BF**	2.38	7.401	orthorhombic	$P2_12_12_1$	[15]

[a] Distance between magnetically nonequivalent nuclei. [b] Identity or inversion characterizes symmetry of sample in the crystal lattice. All phosphorus centers are chemically and magnetically equivalent. [c] (+) and (−)**CF** enantiomers are represented by the same crystallographic system and space group so the P···P distances are exactly the same.

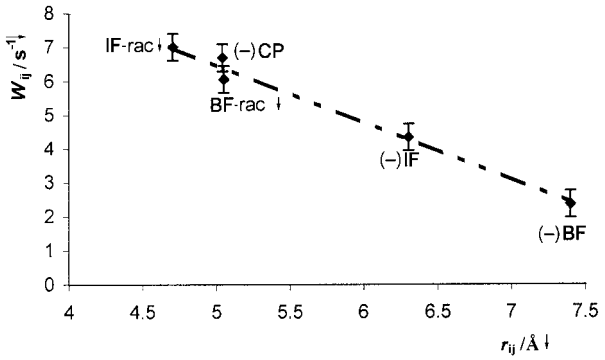

Figure 5. Correlation between decay rates W_{ij} and nearest-neighbor internuclear distances r_{ij} of the enantiomer (−)**CP**, **IF**-rac, (−)**IF**, **BF**-rac, and (−)**BF**.

identical decays to those observed for ground mixtures (Figure 6b). This clearly indicates that under the crystallization conditions used in this work (from ethyl ether to dry sample), racemates and enantiomers of CP form separated phases.

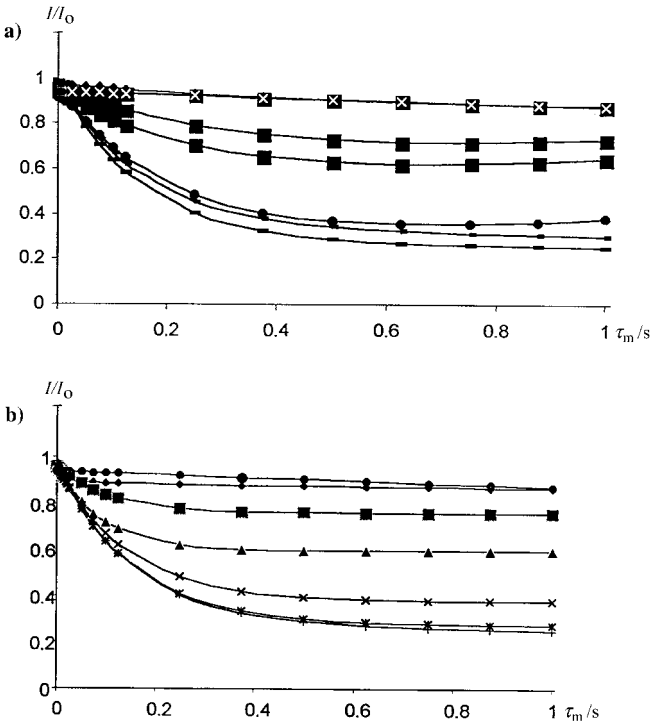

Figure 6. Intensity changes as a function of mixing time for a mixture of **CP**-rac and (−)**CP**. a) ground samples, b) recrystallized samples. The bottom curve corresponds to the racemate, curves in the middle to mixtures with *ee* of 5%, 40%, 50%, 60% and 90%, and the top curve to the pure enantiomer.

A satisfactory correlation (with a regression coefficient of $R^2 = 0.95$) between the amplitude of decay and the enantiomeric excesses, shown in Figure 7, means that the method may be applied to probe the *ee* values. Although in the present case the accuracy in determination of the enantiomeric excess is roughly 10%, one could expect even better precision in systems with shorter internuclear distances and with a small number of exchanging nuclei.

Consequently, the limitation of the proposed approach resides in the fact that only the systems with relatively isolated pairs of abundant nuclei (such as phosphorus or fluorine) or

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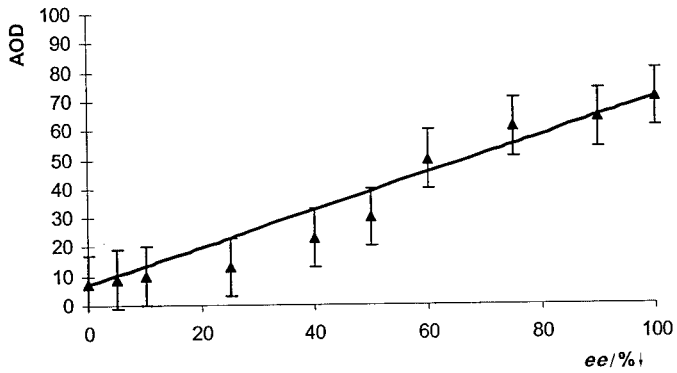

Figure 7. Correlation between the amplitude of the ODESSA decay (AOD) and *ee* for ground mixture of **CP**-rac and (−)**CP**. The final plateau with respect to the initial value (see Figure 6) establishes the amplitude decay.

selectively enriched compounds (*e.g.* ^{2}H, ^{13}C, ^{15}N) can be used safely in such measurements. Moreover, separate powders of pure racemate and enantiomers must be available for calibration purpose.

Conclusion

We have presented a new approach to distinguish between enantiomers and racemates when exploiting P···P distances measurements by high-resolution solid-state NMR. The proposed method also allows the enantiomeric purity of organophosphorus compounds to be probed. Owing to the importance of stereoisomerism for drug efficiency, pharmaceutical companies tend to produce chiral drugs in single enantiomeric forms. Consequently, any new method able to control the enantiomeric purity is greatly to be desired.

Experimental Section

All samples were synthesized according to procedures described previously[10–12] and were recrystallized from ethyl ether shortly before the NMR measurements.

X-ray measurements: The crystal and molecular structure of the **BF** racemate was determined by using data collected at room temperature on a Enraf–Nonius CAD4 diffractometer with graphite monochromatized $Cu_{K\alpha}$ radiation. The compound crystallizes in a monoclinic system, in a centered space group with the unit cell consisting of eight molecules. Crystal data and experimental details are shown in Table 1. The lattice constants were refined by a least-squares fit of 25 reflections in the θ range 18.55°–29.27°. The decline in intensities of three control reflections (−7, −1,1; 3, −3, −3; −5, −3, −3) was 37.5% during 37.5 hours of exposure time, intensity correction was applied (DECAY program).[16] An empirical absorption correction was applied by the use of the ψ-scan method (EAC program).[16, 17] A total of 2460 observed reflections with $F \geq 0\sigma(F)$ was used to solve the structure by direct methods and to refine it by full-matrix least-squares on F^2.[18–20]

Hydrogen atoms connected to carbons were placed geometrically at idealized positions and set as riding with fixed thermal parameters equal to 1.33 times the equivalent isotropic thermal parameter of the parent atom. C–H bond lengths were set to 0.97 Å. The amide hydrogen atom was found on the difference Fourier map and refined with the isotropic thermal parameter. Anisotropic thermal parameters were refined for all non-hydrogen atoms. Both ethylene moieties are disorders in the crystal structure. The occupation factors were refined as equal for atoms in one position and as equal and complementary to one for the second position. Occupation factors for atoms in both disordered ethylene chains are practically equal; this may suggest that both groups changed conformation at the same time.

The final refinement converged to $R = 0.0485$ for 174 refined parameters and 2155 observed reflections with $F \geq 4\sigma(F)$, with inclusion of the extinction parameter into refinement (the obtained value of the extinction parameter was 0.0047(5)).

Data corrections were carried out with the Enraf–Nonius SDP crystallographic computing package,[16] structure solution SHELXS,[17] structure refinement SHELXL.[18]

Solid-state NMR spectroscopy: CP/MAS solid-state ^{31}P NMR spectra were run on a Bruker 300 DSX spectrometer at 121.46 MHz. All spectra were recorded with a contact time of 1 ms, a repetition rate of 6 s, 120 scans were accumulated with high-power proton decoupling. Powder samples were placed in a 4 mm rotor and spun at 2.0–4.5 kHz. ^{31}P chemical shifts were calibrated indirectly through the bis(dineopentoxyphosphorothioyl) disulfide resonance signal set at 84.0 ppm.

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[20] CCDC-181101 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: (+44) 1223-336-033; or deposit@ccdc.cam.uk).

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