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ORIGINAL ARTICLE

Aqueous-phase quantitative NMR determination of amino acid enantiomer ratio by ^{13}C-NMR using chiral neodymium shift reagent

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Abstract A neodymium-(*S*)-PDTA (PDTA = *N*,*N*,*N*′,*N*′-tetrakis[(hydroxycarbonyl)methyl]-1,2-diaminopropane) complex was found exceptionally useful in the quantitative determination of enantiomer ratios of water-soluble natural amino acids by ^{13}C-NMR. The method is demonstrated on mixtures of L- and D-enantiomers of various amino acids. The interactions of the chiral shift reagent with the amino acid molecules were rationalized by molecular orbital calculations.

Keywords Analysis of amino acids · ^{13}C-NMR shift reagent · Neodymium complex chiral shift reagent · Water-soluble chiral shift reagent

Introduction

Fast and easy determination of enantiomeric ratios of free natural amino acids in various aqueous-phase samples of biological and synthetic origin (medical analysis, food quality control, biochemical studies, chemical synthesis, etc.) is one the major problems of amino acid analysis (Pätzold and Brückner 2005; Konno et al. 2007). Chromatographic methods often require specialized instrumentation and are time consuming, especially those which need derivatization. Moreover, some of these methods use non-aqueous solvents (Csapó et al. 1998; Friedman 1999; Simon-Sarkadi 2004; Molnar-Perl 2005).

In the last decades several chiral shift reagents (CSR) containing lanthanide (Ln) ions, complexed by chiral ligands, have been investigated (Gansow et al. 1973; Rothchild 2000; Di Bari et al. 2002, Uccello-Barretta et al. 2006). These reagents, however, are mostly designed for use in non-aqueous solvents. All commercially available products are insoluble in water, except those that are prepared for medical use (NMR imaging) (Magerstadt et al. 1986; Bridot et al. 2007). Some water-soluble Ln-based CSRs were suggested recently, using CSR derivatives of Ce, Eu, Sm (Kabuto and Sasaki 1987; Hulst et al. 1994; Hazama et al. 1996; Ogasawara et al. 1999; Inamoto et al. 2000; Omata et al. 2000; Dickins and Badari 2006). All Ln–CSR complexes used for quantitative determination of enantiomeric excesses (e.e. = $100|(R - S)|/(R + S)$ where R and S are molar quantities of R and S enantiomers of a chiral compound) were applied only in ^{1}H-NMR studies (e.g., Singleton and Vo Loan 2002; Singleton and Vo Loan 2003). One of the major problems with the use of Ln–CSR compounds is that the relaxation times decrease with the consequent loss of fine structure in the ^{1}H-NMR spectra (Morrill 1987). In the last two decade, with the appearance of high-field instruments, this feature has become increasingly inconvenient.

Here we report a fast, versatile ^{13}C-NMR method using a water-soluble CSR, based on Nd, for the determination of e.e.s of water-soluble proteinogenic natural amino acids. Theoretical calculations were also performed for the rationalization of the interaction of this CSR with the amino acids.

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Results and discussion

Analytical results

We found that a suitable water-soluble Ln(CSR) for the problems outlined above, is Nd(III)/(*S*)-PDTA. (*S*)-PDTA (*N*,*N*,*N*′,*N*′-tetrakis-[(hydroxycarbonyl)methyl]-1,2-diaminopropane) can be easily obtained from L-alanine by retention of configuration on C_2 (Florini et al. 2009) and its complex with neodymium can be generated in situ (Dwyer and Garvan 1961; Carr et al. 1967; Kabuto et al. 1992; Inamoto et al. 2000; Aspinall 2002) at pH 10.

We found that Nd/(*S*)–PDTA was particularly useful for the splitting of the resonances (some examples of ^{13}C-NMR spectra of amino acids are shown in Fig. 1 and Table 1) of enantiomers of amino acids in (natural abundance) ^{13}C-NMR spectra (Table 2). Splitting the ^{13}C-resonances was different for the amino acids studied, but in each molecule at least one resonance was split clearly enough to be used for quantitative purposes (Tables 3, 4). This can be best controlled through the integrals of artificially prepared (weighed, ~70 to ~30 mass-%, see Tables 3, 4) mixtures of enantiomers. In some cases, where the ("split") bands were somewhat overlapped, a deconvolution program was used and the integrals gave back the mass ratios of weighed enantiomers very accurately. The linearity of the band ratios was tested for mixtures of D- and L-alanine (Fig. 2). The linearity of this control diagram was tested by linear regression analysis giving $R^2 = 0.999$, indicating an excellent result.

MO calculations

The molecular-level background of the outstanding practical utility of the Nd/(*S*)–PDTA CSR was studied also by density functional (DF) molecular orbital (MO) calculations. The computational details necessary to reproduce our results will be reported later in this paper. DF theory was used to study some of the species relevant to this investigation in order to help clarify the mechanism by which the shift reagent affects the chemical shift of the substrate. Hence, for each species, the geometry was optimized, the energy was recorded and the NMR chemical shift was computed.

Besides providing realistic geometrical descriptions, this also clarified which of the studied isomers were more stable and which would be found in significant amounts under thermal equilibrium conditions. Also, the predicted NMR signal, although generally affected by a significant error bar, was used to establish the trend and the order of magnitude for the reagent-induced shifts.

PDTA coordination

We found two locally stable conformations for the Nd/(*S*)–PDTA complex, labeled (A) and (B) in Fig. 3, corresponding to a different orientation of the methyl group. Conformation (A) was more stable than (B) by 18.8 kJ/mol in vacuum. When water was added as a solvent without relaxing the geometry, this energy difference became 19.7 kJ/mol, indicating that either way the equilibrium population at room temperature would be of the order one molecule of (B) in every 2000 of (A). Thus, the contribution of conformation (B) to the NMR spectra was negligible.

Conformations (A) and (B) are linked to the chirality of PDTA. In fact, the two ligand conformations shown in Fig. 2 have opposite chirality. In order for (B) to relax to the most stable form, the Nd coordination geometry must be changed to the specular image of (A), i.e., the *anti* carboxyl groups must become *gauche* with respect to the next N–C(propane) bond and vice versa. In other words, the relative stability of (A) and (B) reflects how the chirality of PDTA is transferred to metal coordination.

The above results indicate that the interaction of the Nd/(*S*)–PDTA complex with a chiral ligand, such as alanine, would depend on the (chiral, helical) arrangement of the PDTA carboxyl groups around the metal with different enantiomers of the additional chiral ligand coordinating to the same complex with different binding energies and geometries. This is the mechanism by which chiral discrimination occurs. According to this interpretation, we predicted that replacing the PDTA methyl with bulkier groups would have no significant effect on the chiral discrimination properties of PDTA. Such substitution would only make configuration (B) even less stable relative to (A). Since the (B) contribution is already negligible, with the methyl group, this substitution should have no significant additional effect. On the other hand, this grants a certain freedom to replace the methyl with other groups that might be useful for a number of purposes, such as changing the solubility, providing anchors for binding to a support or linking to other ligands, and to attach markers or chromophores, all without changing the coordination geometry around the metal. The computed NMR chemical shifts for the two configurations are sufficiently different to allow a direct experimental confirmation of this result.

Amino acid coordination

Amino acid coordination was studied with the simplest amino acid used in the present study: alanine. For alanine, or rather for $NH_2CH(CH_3)COO^-$, we obtained two locally stable conformations with an energy difference of less than 4 kJ/mol. Since the chemical shifts do not differ by more

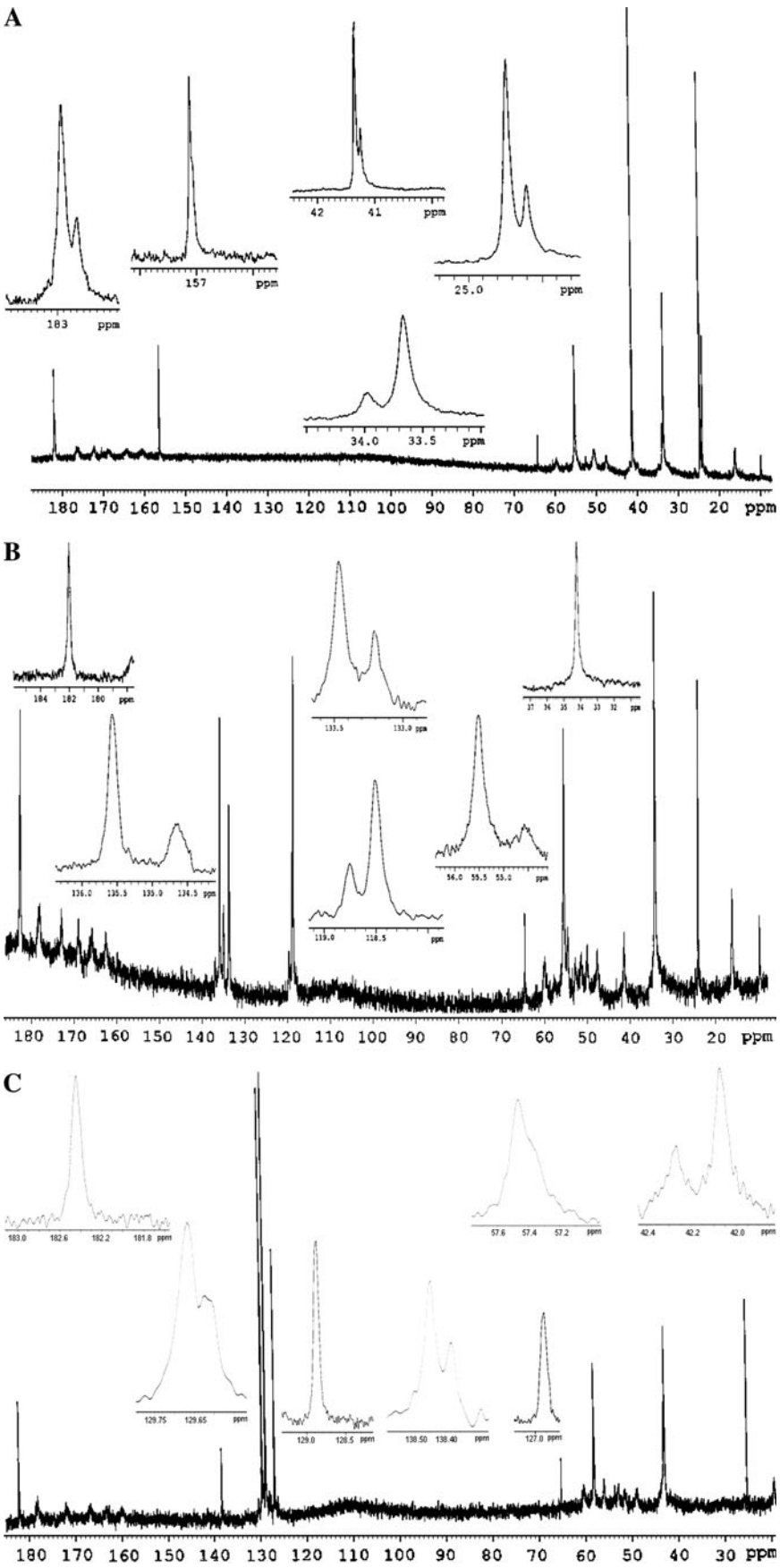

Fig. 1 ^{13}C-NMR spectra of some water-soluble amino acids. **a** Arg, **b** His, **c** Phe

Table 1 ^{13}C-NMR chemical shifts of L-enantiomers of some water-soluble amino acids in the presence of Nd/(*S*)–PDTA

AA	Chemical shifts (δ)						
	COO	αCH	Lateral group				
Ala	181.94	50.88	CH_3: 21.57				
Arg	182.89	55.48	βCH_2: 33.67	γCH_2: 24.48	δCH_2: 41.32	εC: 157.02	
Asn	176.02	49.38	βCH_2: 38.88	CO: 179.62			
His	182.17	55.51	βCH_2: 34.12	γCH_2: 133.47	δCH_2: 118.50	εCH_2: 135.55	
Met	182.74	54.92	βCH_2: 36.50	γCH_2: 29.94	SCH_3: 14.57		
Phe	182.44	57.46	βCH_2: 42.07	Ph: C1: 138.45	C2/C6: 129.67	C3/C5: 128.88	C4: 126.92
Pro	178.49	61.61	βCH_2: 31.11	γCH_2: 24.07	δCH_2: 47.35		

Table 2 Splitting of (natural abundance) ^{13}C-NMR resonances of enantiomers of amino acids in the presence of Nd/(*S*)–PDTA

AA	Splitting ($\Delta\delta$[a])					
	COO	αCH	Lateral group			
Ala	0.296	0.585	CH_3: −0.619			
Arg	0.234	[b]	βCH_2: −0.329	γCH_2: 0.134	δCH_2: 0.095	εC: 0.050
Asn	[c]	−1.22	βCH_2: 1.717	CO: [c]		
His	[b]	0.926	βCH_2: [b]	C1: 0.262	C3: 0.926	C5: −0.53
Met	0.256	0.162	βCH_2: 0.413	γCH_2: 0.139	SCH_3: 0.067	
Phe	[b]	[b]	βCH_2: −0.195	Ph: C1: 0.072,	C2/C6: 0.056, C3/C5[b], C4[b]	
Pro	[b]	−0.223	βCH_2: 0.134	γCH_2: −0.167	εCH_2: 0.067	

[a] $\Delta\delta = \delta_L - \delta_D$

[b] No split bands

[c] Overlapped bands

Table 3 Integrals of the ^{13}C-NMR resonances (natural abundance) of amino acid enantiomers in the presence of Nd/(*S*)–PDTA

AA	Weighed mass L/D ratio (%)	Integrals		
	COO	αCH	Lateral group	
Ala	71.6 28.4	71.6 28.4	71.3 28.7	CH_3: 72.2 27.8
Arg	77.2 22.8	77.1 22.9	[a]	βCH_2: [a] γCH_2: 77.4 22.6 δCH_2: 77.6 22.5 εC: [b]
Asn	75.5 24.5	[b]	70.7 29.3	βCH_2: 75.5 24.5 CO: [b]
His	73.3 26.7	[a]	80.2 19.8	βCH_2:[a] C1: 68.4 31.6 C3:70.1 29.9 C5: 73.2 26.9
Met	74.5 25.5	74.6 25.4	[b]	βCH_2: 74.9 25.1 γCH_2: 72.3 27.7 SCH_3: 69.8 30.2
Phe	63.9 36.1	[a]	[a]	βCH_2: 64.0 36.0 Ph: C1: 63.7 36.4 C2/C6: 62.5 37.5 C3/C5, C4: [a]
Pro	73.0 27.0		76.4 23.6	βCH_2: [b] γCH_2: 72.9 27.1 δCH_2: 73.5 26.5

[a] No split bands

[b] Overlapped bands

than 2 ppm between the two corresponding signals, we assigned an average chemical shift by taking the Boltzmann average of these signals. At room temperature the resulting values (in ppm) were: $C_1 = 171.87$, $C_2 = 56.39$, and $C_3 = 24.14$. When water was added, the main change occurred on the carboxylic carbon (C_1): $C_1 = 179.72$, $C_2 = 57.60$, and $C_3 = 24.14$.

As alanine is coordinated to the Nd-core of the Nd/(*S*)–PDTA complex, several coordination geometries are possible. A number of these involve (bidentate) coordination with both the amino and the carboxylate groups, while many others can be obtained by coordinating just only the amino functionality, in a monodentate manner, to the metal. In this latter case, it would be possible also for a water molecule or an OH^- ion to coordinate to the metal.

In the following part of the present study we were compelled to reduce the huge number of possible models to be considered. Thus, rather than undertaking a systematic

Table 4 Relative errors of the ratios of the best integrals of the ^{13}C-NMR bands with respect to (weighed) mass ratio of enantiomer mixtures of amino acids in the presence of Nd/(*S*)–PDTA

AA	e.e. (L/D)		Relative error (%)
	Mass ratio	Best integral ratio measured	
Ala	43.2514	43.29	−0.089
Arg	54.3699	54.21	0.295
Asn	51.0420	50.92	0.239
His	46.6463	46.24	0.878
Met	49.0610	49.20	−0.282
Phe	27.7969	27.93	−0.476
Pro	45.9922	45.70	0.639

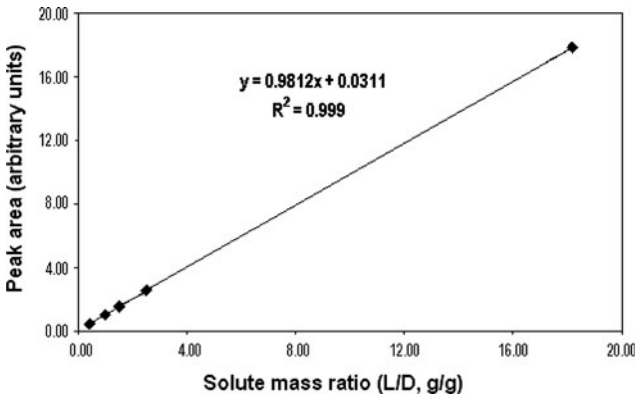

Fig. 2 The linearity of the integral ratio tested for prepared (weighed) mixture of L- and D-alanine

investigation of the very large number of all possible configurations and conformations followed by a Boltzmann average of the corresponding chemical shifts, we selected two representative structures, one for L- and another for D-alanine, with the aim of showing that different coordination geometries do in fact result in different chemical shifts. We stress the fact that due to the arbitrary choice of the two structures this approach cannot be expected to yield an accurate prediction of the experimentally observed signal shift. In fact, our aim was more limited: by showing that two representative structures exhibit different NMR signals with differences of the same order of magnitude as the experimental ones, one provides an argument to sustain that conformational differences such as those proposed can potentially explain the experimental behavior.

Although this argument is certainly not conclusive, it is compelling in its limited scope, in the sense that these computations show that conformational differences can explain shift differences at least as large as those observed. Since, as already mentioned in the previous section, L- and D-alanine are expected to coordinate to Nd/(*S*)–PDTA with different geometries, this would explain the mechanism of chiral discrimination for these species. The results obtained in this study are summarized in Fig. 4.

We have shown in Fig. 4 the computed carbon NMR chemical shifts (ppm) for L-alanine and D-alanine coordinated to Nd/(*S*)–PDTA in each of the two selected conformations. The first values refer to vacuum computations and these are followed in parenthesis by those obtained by addition of water. The differences between the calculated ^{13}C chemical shifts ($\Delta\delta$) of L- and D-alanine coordinated to Nd/(*S*)–PDTA were of the same order of magnitude as the experimentally observed values: $\Delta\delta(C_1) = 0.89\ (0.67)$ ppm, $\Delta\delta(C_2) = 0.20\ (-0.06)$ ppm, and $\Delta\delta(C_3) = 0.11\ (0.30)$ ppm. This result supports the picture described above, how NMR discriminates between different enantiomers.

The computed difference in chemical shifts between alanine in solution and coordinated to Nd/(*S*)–PDTA was much smaller than the experimentally observed value. This could be due to the interaction of the solvated alanine anion with sodium cations (from NaOH) added to the NMR sample in order to raise the pH of the solution. To verify this hypothesis, we computed also the chemical shifts for an alanine donating charge density from its oxygens to a sodium cation. In fact, this interaction resulted in a chemical shift $\delta(C_1) = 186.60\ (187.48)$ ppm. The difference with respect to free alanine was 14.73 (7.76) ppm. For the other carbons, farther from the Na^+ ion, we obtained $\delta(C_2) = 55.54\ (55.58)$ ppm and $\delta(C_3) = 23.58\ (23.83)$ ppm, which are closer to the values computed for free alanine.

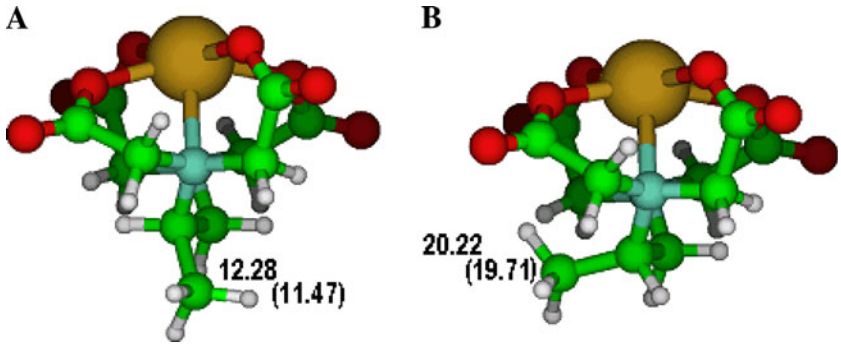

Fig. 3 Two possible conformations of Nd/(*S*)–PDTA

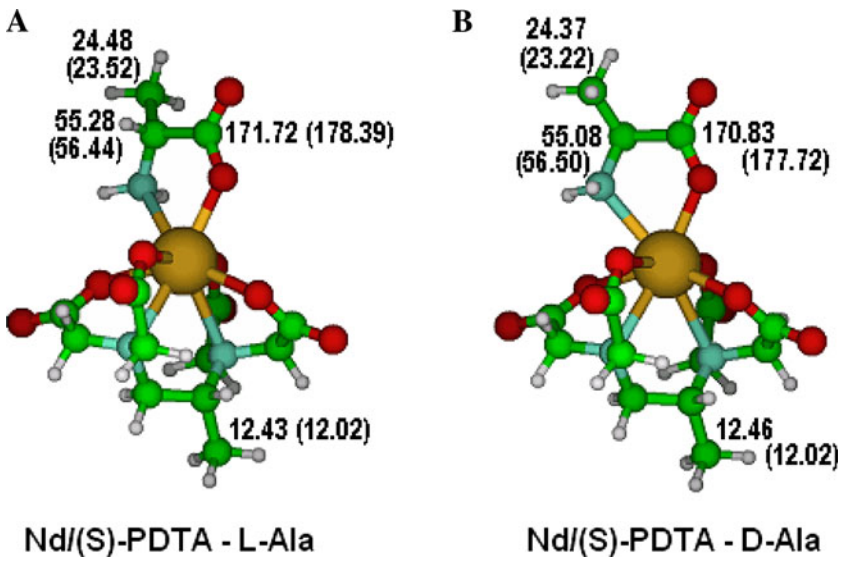

Fig. 4 L- and D-alanine coordinated to Nd/(*S*)–PDTA with different geometries

Next, we considered two alanines interacting with two sodium ions, donating charge both from the oxygens and the nitrogens. In this case the chemical shifts become $\delta(C_1) = 176.60$ (180.64), $\delta(C_2) = 54.78$ (55.88), and $\delta(C_3) = 25.41$ (25.49) ppm. In this case, all carbon chemical shifts are shifted by more than 1 ppm with respect to free alanine. We do not know the exact position of Na^+ ion(s) interacting with each alanine anion and thus we could not provide quantitative estimates of the observed chemical shifts. The trends, however, were the same for the calculated and the experimental values. Based on the computations of Na^+ containing systems, however, we can state that the presence of Na cations in solution will likely result in displacements of the NMR signals of the order of a few ppm.

In conclusion, the computed C-chemical shifts were consistent with the observed NMR spectra and indicated that the mechanism for chiral discrimination is the following. The chirality is transmitted from the PDTA chiral center to the metal via a helical coordinating geometry. Although the chiral carbon does not interact directly with the metal, it determines the orientation of the coordinating carboxylate groups. The orientation of the carboxylate groups results in different binding energies and geometries for L- and D-alanine, which in turn determine different chemical shifts.

The chemical shifts of alanine in solution cannot be calculated exactly, due to the presence of sodium cations. We showed, however, that changes in the band position by a few ppm are plausible upon coordination to the Nd/(*S*)–PDTA CSR. The differences between coordination of L- and D-alanine, instead, are computed in the range of tenths of a ppm.

Conclusions

We found that neodymium(III) complex of (*S*)–PDTA can be used as band splitting agent in the quantitative enantioselective analysis of the ratio of enantiomers of water-soluble natural amino acids by ^{13}C-NMR spectroscopy. Beyond the excellent band splitting effect of the Nd(III)/(*S*)–PDTA reagent some other advantages include:

a. The much lower quantities of the CSR which were required for analysis in comparison with the Sm-based analog (by a factor of ~ 10).
b. Considerably shorter acquisition times were necessary than for spectra obtained without additive (effect on relaxation). The sequence used and the following spectra interpretation was a routine process level.
c. The method offers the possibility to perform measurements in mixtures of $H_2O/D_2O \sim 3{:}1$–$5{:}1$.

Quantitative use of ^{13}C-NMR spectra is generally hindered by slow relaxation of ^{13}C-nuclei; therefore, the use of our Nd-based CSR as relaxation agent is particularly useful also from this aspect, since the broader range of the ^{13}C-NMR spectra (with respect to the ^{1}H-spectra) provides better quality (less overlapping) and this advantage can be exploited through the use of the reagent described in the present work.

The method described here has a general advantage from that point of view that the signals of the same group in the two enantiomers are submitted to very close relaxation times in the NMR experiment and therefore their quantitative comparison *should* give reliable data (as demonstrated also experimentally by us). This comparison would not be possible with the same precision for different

groups in ^{13}C-spectra. The use of ^{13}C-spectra is particularly advantageous (over ^{1}H-spectra) in aqueous phase, since the ^{1}H-spectra are much more sensitive than the ^{13}C-spectra even to minor variations in the pH.

It appears reasonable to believe that the results reported in the present paper, considering the viewpoints listed above, give a good basis for very easy and fast enantiomeric analysis of free amino acids either alone or in mixtures with others in aqueous phase. MO–DF calculations have shown that the signal splitting mechanism is proceeding from the configurational chiral center in the PDTA ligand through conformational chirality of its coordination to the metal, which, on the other hand, results in energy differences (and consequently different NMR signals) of enantiomers of the amino acid to be analyzed. The effect of water molecule(s) and the presence of Na^{+} ions (the latter used for adjusting the pH) could also be rationalized by model DF calculations.

Experimental

Reagents were of commercial origin except the (*S*)-PDTA ligand, which was prepared as described earlier (Florini et al. 2009). Measurements were performed with a Bruker FT-NMR Avance 400 MHz spectrometer using 100.62 MHz for the ^{13}C measurements. The ^{13}C-NMR spectra were obtained using a zgpg (standard decoupling sequence and NOE growth during D1), with the following acquisition parameters: 90° pulse length 13.90 μs with 16k data point, spectral width 200 ppm, scan number 4k, receiver gain 32k and D1 250 ms. All spectra are zero-filled with 32k data point and have 2 Hz of line broadening. T1 determination was performed with a t1irpg sequence and using the following acquisition parameters: 90° pulse length 13.90 μs with 16k data point, spectral width 200 ppm, scan number 128, receiver gain 32k and D1 25 s using 11 vdlist values applying the automatic fitting program of XWIN-NMR of Bruker. Masses of amino acid samples for the "artificial mixtures" of enantiomers were measured (weighed) by a Sartorius Research R 200 D balance, which guarantees 0.01 mg masses as significant figures if the sample mass is less than 42 g (as it was always in the present study). The masses of the samples measured were in the range of 50–100 mg.

Sample preparation

0.101 g (0.268 mmol, 0.68 eq) of H_4PDTA × 2HCl, and amino acid (0.4 mmol, 1 eq) are fed in a test tube with (in the following order) 200 μL D_2O, 300 μl of H_2O, 400 μL of 10 M NaOH (in H_2O), and 0.040 g (0.16 mmol, 0.4 eq) of $NdCl_3$. Normally a clear solution is obtained. If a precipitate was formed the pH was adjusted to 1 by the addition of 3 M HCl and then the addition a 10 M NaOH solution was necessary until pH 10.3 was reached.

Computational details

All computations were performed using the Gaussian03 program suite (Gaussian 03, Revision C.02, Frisch et al. 2004) using the Becke3LYP (Becke 1993; Lee et al. 1988; Miehlich et al. 1989) DF. The basis set used for the light atoms was 6–31G** (Hariharan and Pople 1973) with diffuse functions (Clark et al. 1983) added to the oxygens. To describe the core electrons of Nd we used the Effective Core Potential (Cundari and Stevens 1993) and basis set.

Where indicated, the solvent effect of water was treated with the polarized continuum model (Miertus and Tomasi 1982; Cancès et al. 1997; Barone et al. 1998) at the geometry optimized in vacuum. NMR shielding tensors and the corresponding chemical shifts were computed using the GIAO formalism of magnetic response theory (Wolinski et al. 1990).

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