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Direct Chiral HPLC Separation of the Enantiomers of Fluorinated N-arylamino-1-arylmethylphosphonate Esters. Substituent Effects on the Enantioselectivity

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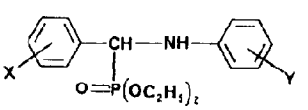
Abstract: Racemic N-arylamino-1-arylmethylphosphonic acid diethyl esters with various fluorinated substituents in one or both aryl rings have been resolved in their enantiomers by a direct HPLC method, using chiral stationary phases. The chiral separation on Chiralcel OD strongly depends on the substitution pattern in the N-aryl and/or in the C-aryl moieties and it improves markedly with the polarity of the fluorinated substituent. A new Pirkle's chiral stationary phase (R)- α-Burke 1 is more efficient and in some cases more enantioselective than other Pirkle's phases. A chiral recognition model between this phase and the enantiomers of an analyte afforded to propose the absolute configuration of the optical isomers and to relate it to the chiroptical behaviour.

α-Aminophosphonic acids are bioisosters of natural aminoacids and are widely used to modify biological processes. They are used as enzyme regulators,[1] antifungal agents[2] and in the preparation of phosphonodipeptides clinically studied as antibiotics.[3] The biological activity of α-aminophosphonic acids depends on the configuration at the chiral center as reported in several literature cases. Recently, as an example, condensation of racemic phosphonate esters with vinblastine gave epimers which show very different antitumor activity depending on the stereochemistry of the phosphonate.[4] Single enantiomers are usually obtained by resolution of racemic mixtures through the formation of diastereoisomeric salts[5] or diastereoisomeric phosphonodipeptides[3a,6] and subsequent hydrolytic cleavage. Few efficient asymmetric syntheses of enantiomerically enriched α-aminophosphonic acids have recently been reported.[7]

Recently increasing interest has been devoted to the synthesis of fluorinated α-aminophosphonic acid derivatives, since fluorine leads to a strong polarization of the molecules and as a consequence increases their biological activity. In this context many C-fluorinated aromatic α-aminophosphonic acids were obtained.[8] Some of us has dedicated some efforts to to the synthesis, characterization and molecular modelling of N-arylamino-1-arylmethylphosphonic acid derivatives bearing fluorine-containing substituents.[9] Compounds investigated in this paper belong to the general formula shown below



X=H, 3,4-F_2, 4-OCF_3 Y=H, 2-CF_3, 2-OCF_3, 2-F, 3-F, 4-F, 3-CF_3 4-CF_3, 3,4-F_2

and were previously obtained as racemic mixtures. Remarkably, screening on these for the inhibition of the mitochondrial NADH dehydrogenase indicated strong activity for compound **99a** (see Table 1).

Recently some of us reported a direct and efficient HPLC enantioselective resolution of diethyl N-phenylamino-1-phenylmethylphosphonate[5] using chiral stationary phases (CSP). The present work deals with the direct HPLC resolution of almost thirty new fluorinated diethyl N-arylamino-1-arylmethyl phosphonates using as CSP Chiralcel OD and in some cases Pirkle's CSP. A chiral recognition model between this phase and compound **27** suggested the absolute configuration of the enantiomers and its relationship to the chiroptical behaviour.

For one of the best resolved compounds (X=Y=4-CF_3) we performed a 20 mg separation of the individual enantiomers and enzymatic inhibition testing on each of them. This activity was however modest and almost the same for both enantiomers.

The substitution pattern in the N-aryl and C-aryl moiety strongly influences the enantioselectivity and, in general, the chiral separation on Chiralcel OD phase markedly improves with the substitution in the *para* position of the N-aryl group and with the increasing polarity of the substituent.

Results and Discussion

Table 1 shows the chromatographic results for the enantioseparation of compounds **1–28** using as chiral stationary phase Chiralcel OD *without effecting any derivatization*. Enantioselectivity (α) and resolution factor (R_S) range from 1.86 and 3.1 respectively for compound **27** to 1.13 and 0.8 for compound **12**, using as eluent *n*-hexane/2-propanol 9:1. Moreover, the separation factors α are almost unaffected by the percentage of 2-propanol in the mobile phase. A decrease in the polarity of the mobile phase was critical only to obtain separation in compounds **4**, **7** and **10**.

The most important observation from the results in Table 1 is the beneficial effect on the enantioselectivity of the *para* substitution in both aryl rings. Compounds that differ only for the position of the substituent in the ring (*ortho, meta or para*) behave very differently with respect to their enantiomeric separation, as shown by the comparison of the isomers **2–4** and **6–8**, **9** and **10**, **18** and **19** and in the chromatograms reported in Figure 1.

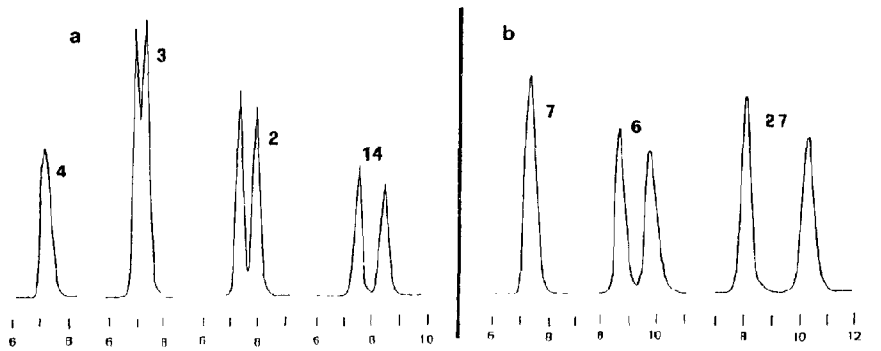


Figure 1
CSP HPLC behaviour of the pair of enantiomers of compounds **4**, **3**, **2**, **14** (a) and **7**, **6**, **27** (b). Mobile phase *n*-hexane/2-propanol 9:1 (a) and 95:5 (b).

Table 1. HPLC Resolution of Enantiomeric Compounds **1–28** on Chiralcel OD

Comp.	X[a]	Y[a]	%A[b]	k'[c]	α	R_S	Comp.	X[a]	Y[a]	%A[b]	k'[c]	α	R_S
1	H	H	10	0.66	1.24	1.1	**12**	3-F	H	10	0.72	1.13	0.8
			5	1.05	1.27	1.7							
							13	2-F	H	10	0.58	1.38	1.7
2	H	4-F	10	0.61	1.22	1.0							
			5	1.06	1.23	1.5	**14**	4-F	4-F	10	0.59	1.35	1.6
3	H	3-F	10	0.51	1.14	0.8	**15**	4-F	4-CF_3	10	0.37	1.86	2.1
			5	0.83	1.15	0.8							
			1	2.81	1.16	1.4	**16**	4-F	4-OCF_3	10	0.44	1.57	2.4
4	H	2-F	10	0.59[d]	NS[e]		**17**	3,4-F_2	H	10	0.74	1.15	0.8
			5	0.89	1.06	0.5							
			1	2.35	1.11	1.0	**18**	3,4-F_2	4-F	10	0.63	1.38	2.2
5	H	3,4-F_2	10	0.52	1.16	0.6	**19**	3,4-F_2	3-F	10	0.55	1.26	1.2
6	H	4-CF_3	10	0.49	1.25	1.0	**20**	3,4-F_2	3,4-F_2	10	0.62	1.29	1.4
			5	0.86[d]	1.27	1.3							
							21	3,4-F_2	4-CF_3	10	0.52	1.59	2.5
7	H	3-CF_3	10	0.38	NS		**22**	3,4-F_2		10	0.45	1.72	2.8
			5	0.56[d]	NS								
			1	1.47	1.08	0.6	**23**	4-OCF_3	4-F	10	0.48	1.52	2.3
8	H	2-CF_3	10	0.48	1.20	1.0	**24**	4-OCF_3	4-CF_3	10	0.41	1.65	2.6
			5	0.86	1.24	1.3							
9	H	4-OCF_3	10	0.45	1.35	1.4	**25**	4-OCF_3	4-OCF_3	10	0.34	1.83	2.6
			5	0.84	1.35	1.8							
							26	4-CF_3	4-F	10	0.60	1.57	2.6
10	H	2-OCF_3	10	0.44[d]	NS		**27**	4-CF_3	4-CF_3	10	0.47[f]	1.68	2.8
			5	0.71	1.16	0.8				5	0.74	1.69	3.1
			1	2.07	1.21	1.5							
11	4-F	H	10	0.64	1.22	1.1	**28**	4-CF_3	4-OCF_3	10	0.37	1.86	3.1
										5	0.70	1.83	3.6

[a] According to the general formula in the text. [b] OD: Chiralcel OD; GEM: (R,R)-β-GEM1. [c] Percent of 2-propanol in *n*-hexane at a flow rate of 0.7 ml/min, t_0=4.62 min in OD, 4.12 min in GEM. [d] Capacity factor of the first eluted enantiomer. [e] Shoulder in the rising edge of the peak. [f] Not Separated. [g] CD positive enantiomer, see text.

Moreover, chiral separation improves markedly with the polarity of the substituent in the *para* position of the C-aryl ring for the same *para* substituent in the N-aryl ring, as shown in the curves of Figure 2a or in the ordinate scale of Figure 2b, taking into account the dipole moment of the substituent (-1.47, -2.21, -2.36 and -2.54 for 4-F, 3,4-F_2 free rotating, 4-OCF_3 and 4-CF_3 respectively).[11] A similar trend is observed when, for the same X-*para* substituent, the polarity of the Y-*para* substituent is increased as presented in the four curves of Figure 2b where fifteen compounds are considered. An inversion between $-CF_3$ and OCF_3 is observed, probably due to the lack of rotation of the N-aryl conjugated group and to the angle formed by the OCF_3 dipole moment with the 1,4 axis (160°).[11]

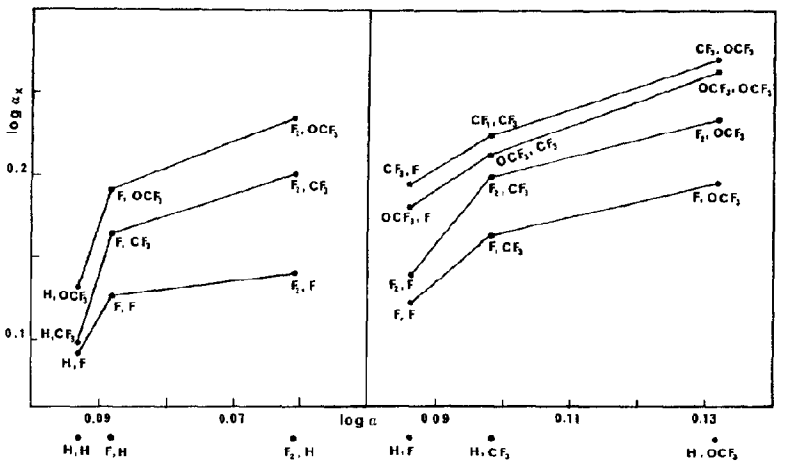


Figure 2
The curves represent the log α_X where x is the varying substituent in 4-X position (a) and in 4-Y position (b). X, Y refer to *para* substituents in the general formula in the text. Log α of reference compounds, where H is in 4-Y position (a) and in 4-X (b) position, are reported in the abscissa.

Thus very fine substituent effects tune the interaction of the enantiomers of these N-arylamino-1-arylmethylphosphonates with the polar carbamate moiety of the Chiralcel OD phase. This phase owes its chirality to the macromolecular helicity but it is clear, from the above results, that the strength of the dipole-dipole stacking[12] between the π-donor 3,5-dimethylphenyl carbamate group pending from the glucose rings of the phase and the π-acceptor phenyl substituted rings of the analyte is crucial for efficient chiral discrimination. This effect, if found for other classes of *isomeric compounds*, has to be taken into account when approaching a chiral separation with this phase.

Trying to improve the separation factor of the compounds which are scanty resolved on Chiralcel OD phase, we checked the chromatographic behaviour of some of them on two new Pirkle-type columns. One of them (α-Burke 1) possesses a phosphonate ester moiety[13], the other one possesses two stereogenic centers (R,R β-GEM 1).[14] The results are indicated in Table 2.

Table 2. Pirkle's CSPs-HPLC Resolution of Selected α-Aminophosphonate Esters.

Comp.	Xa	Ya	CSPb	k'c,d	α	R_S
1	H	H	A	1.07	1.14	1.4
			B	2.36	1.11	0.8
9	H	4-OCF_3	A	0.63	1.18	1.2
			A	0.92e	1.20	2.4
			A	0.65e,f	1.20	1.5
			B	1.27	1.05	0.6
10	H	2-OCF_3	A	0.57	NSg	
			B	1.53	1.07	0.7
11	4-F	H	A	1.07	1.12	1.1
			B	2.12	1.11	0.9
17	3,4-F_2	H	A	1.13	1.22	2.2
			B	2.09	1.11	0.8
27	4-CF_3	4-CF_3	A	0.80	1.20	1.5

[a] According to the general formula in the text.[b] Chiral Stationary Phases, A=(R) α-Burke 1 and B=(R,R) β-GEM1.
[c] Capacity factor. [d] *n*-hexane/2-propanol 9:1 at a flow rate of 0.7 ml/min., t_0=4.32 min., unless otherwise specified.
[e] *n*-hexane/2-propanol 95:5. [f] Flow rate at 1 ml/min., t_0=3.53 min. [g] Not separated.

Compounds **9** and **17** show increased chiral resolution on the α-Burke phase with respect to the Chiralcel OD. For all the examined cases the former phase is far superior to the β-GEM phase, indicating that the interaction between the phosphonate ester moieties of the CSP and of the analyte not only increase the efficiency of the column but improves also, as expected[13], the chiral recognition process. The dramatically different performances of three Pirkle's CSP on the chiral separation of compound **17** are shown in Figure 3 and they can, in fact, be rationalized by the different chiral recognition processes through the three binding sites model indicated by Pirkle.[15]

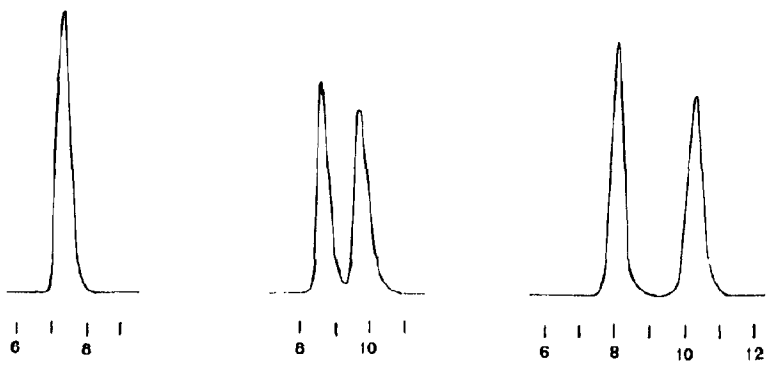


Figure 3
HPLC behaviour of the pair of enantiomers of compound **17** with three Pirkle CSP: (R)-DNBPG, (R,R)-β GEM1 and (R)-α-Burke 1. Mobile phase *n*-hexane/2-propanol 9:1 at flow 0.7 ml/min.

Compounds **9**, **10**, **11**, **16** were also chromatographed using *n*-hexane/2-propanol 9:1 on the classical

Pirkle DNBPG column but only a sharp peak was obtained.

The excellent resolution factor obtained for compound **27** afforded a separation of its enantiomers by repeated 50-μl injection of racemic **27** and collection of the eluates from the chromatographic peaks. The CD spectra of both eluates were measured and they were mirror images of each other as shown in Figure 4, indicating that the two eluates are optical isomers. Analytical HPLC reruns of the eluates indicated an enantiomeric purity of 99 % for the first peak and 80 % for the second peak. Their UV spectra were also identical.

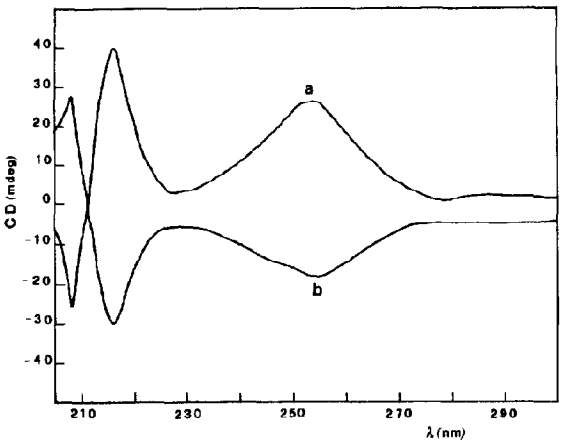


Figure 4. CD spectra of the pair of enantiomers of compound **27** obtained from the first (a) and the second (b) HPLC eluted peaks.

The isolated enantiomers were then chromatographed on the α-Burke CSP and the elution order on this column was the same as on Chiralcel OD.

Since we know the absolute configuration (R) of the CSP, taking into account the most stable conformations of the CSP and of the analyte **27**, we built a recognition Dreiding model with the enantiomers. A hydrogen bonding mechanism with the (S) analyte held most strongly by the CSP is shown in Fig. 5. In the case of the heterochiral (i. e. the R:S) complex hydrogen bonding interactions between the -P=O of the CSP and -NH group of the analyte and *viceversa* lead to a structure where the analyte's C-aryl substituent is presented far from the N-aryl and phosphonate groups of the analyte and a ten membered ring is formed, as shown by the dotted lines in Fig. 5. To maintain the pivoting hydrogen bonding interaction, the R enantiomer is instead forced to an unfavourable orientation where the resulting steric difficulties of the C-aryl substituent reduce retention.

Thus, we propose that the CD positive enantiomer of compound **27** has R configuration.

The hypothesis of the transient diastereomeric complex *via* two hydrogen bonds formation agrees with two observations supporting the strength of the -P=O····H-N hydrogen bond. First, an X-ray diffraction analysis[16] showed that racemic compound **1** forms centrosymmetric hydrogen-bonded dimers; second, the FAB(+) mass spectra of compounds **1** and **27** show a significant presence of dimeric forms probably through hydrogen bond formation. Compound **1** shows in fact, beside the base peak $[M-P(O)(OC_2H_5)2]^+$ at m/z 182, ions at m/z 320 (rel. ab. 9.1) due to $[M + H]^+$, m/z 501 (0.3) due to $[2M - 137]^+$, m/z 639 (2.2) due to $[2M+H]^+$

Analogously, compound **27** shows, beside the base peak at m/z 318 due to $[M - 137]^+$, ions at m/z at 455 (9.6) due to M^+, m/z 773 (0.2) due to $[2M - 137]^+$, m/z 911 due to $[2M+ H]^+$

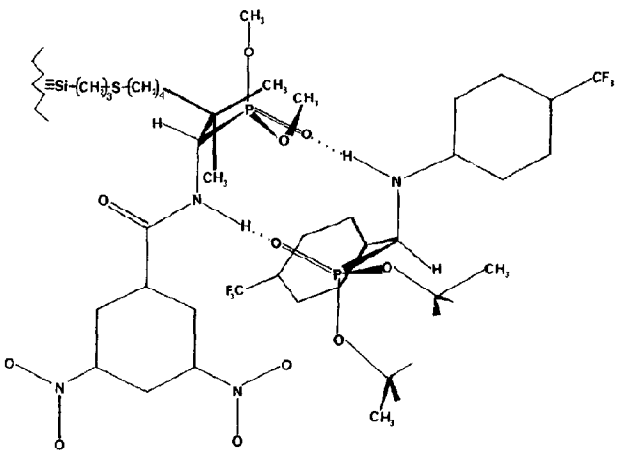


Figure 5. Molecular model representation of the diastereomeric adsorbate between (R) α-Burke 1 phase and (S) compound **27**.

The determination of the inhibitorial activity at the NADH dehydrogenase in the mitochondria of *Neurospora crassa* of the enantiomers 1 and 2 of compound **27** (1st and 2nd eluted peak respectively), as well of the racemic compound, was performed according to an established procedure.[16] The respiratory-chain NADH dehydrogenase of mitochondria is one of the most complex among the proton translocating enzyme and interest in it has recently increased.[17] Thus, we wanted to investigate if chiral discrimination from the enzyme was effective. This was not the case, however. In fact, $K_{inhibition\ 50}$ were 54.7, 38.5 and 42.0 μg/mg protein for enantiomers 1 and 2 and racemate, respectively. These values are very close and complete inhibition of the enzyme was not reached also using high concentration of the compounds. Thus, in comparison with the complete inhibiting compound **9** (racemic form)[9a], compound **27** as racemate or pure enantiomers is not an useful inhibitor.

Experimental

The syntheses of the compounds **1–28** are described elsewhere.[9b] They were synthesized by addition of diethyl phosphite at 80–100 °C to the suitable benzylidineamine which was obtained by reaction of the appropriate X-bearing benzaldehyde with the appropriate Y-bearing aromatic amine. The HPLC system consisted of a Varian 5060 liquid chromatograph with Valco sample loops, a Jasco Uvidec III UV spectrophotometric detector operating at 240 nm, and a Varian CDS 401 Data System or a Omniscribe Houston recorder for fraction collecting. CD spectra were recorded on a Jasco 600 Spectropolarimeter. The mobile phases were HPLC-grade *n*-hexane/2-propanol mixtures. The columns (25 cm x 4,6 mm) were packed with Chiralcel OD (Cellulose tris-3,5-dimethylphenylcarbamate) coated on 10 um silica gel, from Daicel (Tokyo) and with (R)-α-Burke **1** [dimethyl N-3,5-dinitrobenzoyl-α-amino 2,2-dimethyl-4-

pentylphosphonate] covalently bonded to 5 μm mercaptopropylsilica, (R,R) β-GEM 1 [N-3,5 dinitrobenzoyl-3-amino-3-phenyl-2(1,1-dimethylethyl)-propanoate] covalently bonded to 5 μm silica gel and (R)-DNBPG (N-3,5 dinitrobenzoylphenylglycine) covalently bonded to γ-aminopropylsilanized silica, all of three from Regis Chemical (Morton Grove, IL). Column void time (t_0) was measured by injection of tri-*tert*-butylbenzene as a nonretained sample. Retention times were mean values of two replicate determinations. All separation were carried out at ambient temperature. FAB + mass spectra were obtained on a VG ZAB 2 SE instrument, using as matrix 3-nitrobenzylalcohol.

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