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Use of L-Lysine Fluorescence Derivatives as Tracers To Enhance the Performance of Polarization Fluoroimmunoassays. A Study Using Two Herbicides as Model Antigens

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Fluorescence polarization immunoassay (FPIA) is a convenient homogeneous assay, the use of which is restricted in environmental analysis by low sensitivity and matrix effects. We selected the herbicides 2,4D and 2,4,5T to synthesize new L-lysine-based fluorescent tracers using solid-phase chemistry. In addition, three different immunogens of 2,4,5T were prepared for immunization and antibody production. The new tracers and antibodies were adapted to FPIA. Tracers with the hapten attached to the α-aminogroup of L-lysine and fluorescein to the e-amino group exhibited at least a 5-fold increased sensitivity when compared to the previously reported ethylenediamine-based tracer (2,4D-EDA-F). The isomeric structure (hapten attached to the e-amino and fluorescein to the a-amino group) appeared 7.6 times less sensitive, and all other alternative structures exhibited even lower sensitivities. This observation was confirmed against the monoclonal anti-2,4D antibody E2/G2 and polyclonal anti-2,4,5T antibodies. The affinity constant of 2,4D-EDA-F with E2/G2 was 8.1 times higher when compared with the new tracer, suggesting the more specific nature of the L-lysine-based tracer, the use of which leads to a more sensitive assay. This type of tracer could improve performance and lower substantially the detection limits of FPIAs.

A large number of heterogeneous and homogeneous immunoassays have been developed for detection of biologically active residual compounds responsible for food and environmental contamination. They include ELISA with colorimetric, chemiluminescent, or electrochemical detection, biosensors and immunosensors, flow-injection immunoassays, and fluorescence immunoassays.[1−6] These are used for direct detection of the analyte or for screening purposes.[7] Among these, the homogeneous methods are fast, simple, and inexpensive and they do not require separation or washing steps.

The fluorescence polarization assay (FPIA) is the most widely used homogeneous assay with applications mainly in therapeutic and drug abuse monitoring.[8−10] In recent years a lot of work has been performed for FPIA development and application in environmental analysis. Pesticides such as 2,4D, 2,4,5T, atrazine, simazine, isoproturone, methabenzthiazurone, dichlorprop, and propazine have been used as antigens.[6,8,11,12] Despite the apparent advantages, the relatively low sensitivity remains as the major limitation of the assay.[8] This is particularly important for residue analysis where the limits of detection (LODs) are very low and the sample matrixes are complex. Efforts for further FPIA optimization are directed toward improvement in sensitivity with the use of new more suitable materials and fluorescent dyes,[13,14] new instruments,[8] and new and alternative methodologies such as 2-PEFA (2-photon excited fluorescence anisotropy)[15] or stopped-flow FPIA,[16,17] where the matrix effect is minimized.

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The specific antibody used is one of the key components of FPIA, like in any other immunoassay, that determines the quality of the method.[18−20] In FPIA, however, the tracer structure also greatly affects the sensitivity. Tracers of 2,4D with different bridge lengths (2, 4, or 6 carbon atoms) have been compared using the same antibody. The most sensitive assay was obtained using the ethylenediamine (2 carbon atom chemical bridge) based tracer.[8,21] Similar findings have been reported for atrazine FPIA.[6,22] Therefore these kinds of tracers have been used for FPIA optimization.[16,17,23]

In this work, we used the herbicides (2,4-dichlorophenoxy)-acetic acid (2,4D) and (2,4,5-trichlorophenoxy)acetic acid (2,4,-5T) as model analytes and studied the influence of tracer structure in FPIA characteristics, mainly sensitivity. 2,4D is a widely used herbicide that could potentially contaminate the ground and water and enter the food chain. The presence of dioxin traces in 2,4,5T has led to its withdrawal from the market and its use forbidden. The importance of this molecule remains high because monitoring of 2,4,5T levels serves as a potential indicator of dioxin pollution.[24] We introduced L-lysine as a spacer for preparation of monolabeled and bilabeled fluorescent tracers of 2,4D and 2,4,5T by applying solid-phase chemistry. In addition, three different bridge length 2,4,5T immunogens were synthesized. The monoclonal antibodies clone E2/G2,[25] specific for 2,4D and all polyclonal antibodies produced against 2,4,5T immunogens, were adapted to FPIA in combination with the new tracers. The corresponding ethylene-diamine-based tracers were also used for comparison purposes. The results of these assays are presented in this study, indicating that a new particular L-lysine-based tracer structure is important in improving further the FPIA sensitivity.

EXPERIMENTAL SECTION

Materials. 2-Chlorotrityl chloride resin (CLTR) (1.5 mmol of reactive Cl/g of resin) and all Fmoc-protected amino acids, Fmoc-K(Boc)-OH, Fmoc-K(Fmoc)-OH, Fmoc-K(Mtt)-OH, Fmoc-βAlα-OH, and Fmoc-ϵAca-OH, were purchased from CBL, Patras, Greece. Dicyclohexyl carbodiimide (DCC), hydroxybenzotriazole (HOBt), *N*-hydroxysuccinimide (NHS), and all other chemicals and organic solvents were purchased from Merck (Darmstadt, Germany). (2,4-Dichlorophenoxy)acetic acid (2,4D) (UV_{max} = 284 nm, ϵ_{1M} = 2100) and (2,4,5-trichlorophenoxy)acetic acid (2,4,5T) (UV_{max} = 289 nm, ϵ_{1M} = 2550)[26] of analytical grade and fluorescein 5-isothiocyanate isomer I (FITC) were purchased from Sigma. (2,4-Dibromophenoxy)acetic acid (2,4DBr) and other structurally related compounds for cross-reaction studies were provided from Riedel-de-Haen (Hannover, Germany). Monoclonal anti 2,4D antibodies (E2/G2) were obtained at the VRI (Brno, Czech republic) as elsewhere described[25] and kindly provided by Dr. Milan Franek. All reagents were of analytical grade. The water in all experiments was double distilled water, and the buffer for FPIA was the TDx buffer of Abbot Diagnostics. Stock solutions of analytes and cross-reactants were prepared in methanol. TLC for analysis and purification of products and ninhydrin tests was executed as elsewhere described.[27] The TLC solvent system in all cases was 7:1.5:1.5 toluol−methanol−acetic acid.

Abbreviations: Fmoc, fluorenylmethyloxycarbonyl; Boc, butyl-oxycarbonyl; Mtt, methoxytrityl; βAla, β-alanine; ϵAca, ϵ-amino-caproic acid; K, L-lysine.

Synthetic Procedures. The synthetic process we followed included (a) synthesis of L-lysine and ethylenediamine (EDA) derivatives of the analytes, using 2-chlorotrityl chloride solid-phase chemistry, (b) labeling of all derivatives with FITC for tracers preparation, and (c) synthesis of 2,4,5T haptens with solid-phase chemistry and preparation of immunogens.

*One Lysine Molecule Resin, H_2N-K(Boc)-CLTR (**1**), and Two Lysine Molecule Resin, H_2N-K_2(Boc)$_2$-CLTR (**2**)*. The very acidic labile CLTR resin was used as the solid phase. Synthesis started with 1 g of CLTR resin and 280 mg (0.6 mmol) of Fmoc-K(Boc)-OH, and the well-established protocol for CLTR loading was followed.[28] The yielded resin had a substitution of 0.4 mmol of Fmoc-K(Boc)-OH/g of CLTR. It was then treated with 25% piperidine solution for 30 min to remove the Fmoc group and washed successively with dimethyl formamide (DMF), 2-propanol, and ether. The resulting resin H_2N-K(Boc)-CLTR (**1**) was dried in a nitrogen stream.

A 300 mg amount of resin **1** used for preparation of resin **2**. A 170 mg (0.36 mmol) amount of Fmoc-K(Boc)-OH in 3 mL of DMF and DCC/HOBt (82 mg/82 mg) as the condensing agent was used[28] in a 2 h reaction with resin **1**. The completion of the reaction was checked with TLC.[27] Resin wash and Fmoc removal as above were followed. The resulting resin H_2N-K_2(Boc)$_2$-CLTR (**2**) was dried in a nitrogen stream.

*2,4D-K(NH_2)-OH (**3a**), 2,4,5T-K(NH_2)-OH (**3b**), 2,4D-K_2-(NH_2)$_2$-OH (**4a**), and 2,4,5T-K_2(NH_2)$_2$-OH (**4b**)*. A 100 mg amount of each resin **1** and **2** with approximately 0.04 mmol of amino groups each was used for reaction with 0.2 mmol of 2,4D and 2,4,5T. The condensing agent NHS/DCC (26 mg/46 mg) was used for a 1 h reaction of herbicides with resins **1** and **2**. After the usual washes, resins were treated for 30 min with 1 mL of 90% TFA to cleave haptens and simultaneous Boc deprotection. The filtrates evaporated in nitrogen stream and the products 2,4D-K(NH_2)-OH (**3a**), 2,4,5T-K(NH_2)-OH (**3b**), 2,4D-K_2(NH_2)$_2$-OH (**4a**), and 2,4,5T-K_2(NH_2)$_2$-OH (**4b**) (Figure 1) were easily isolated with diethyl ether precipitation, centrifugation, and drying. The ninhydrin test on TLC plates in all cases was positive, R_f**3a** = 0.2, R_f**3b** = 0.25, R_f**4a** = 0.01, and R_f**4b** = 0.02. UV_{max} of **3a** and **4a**

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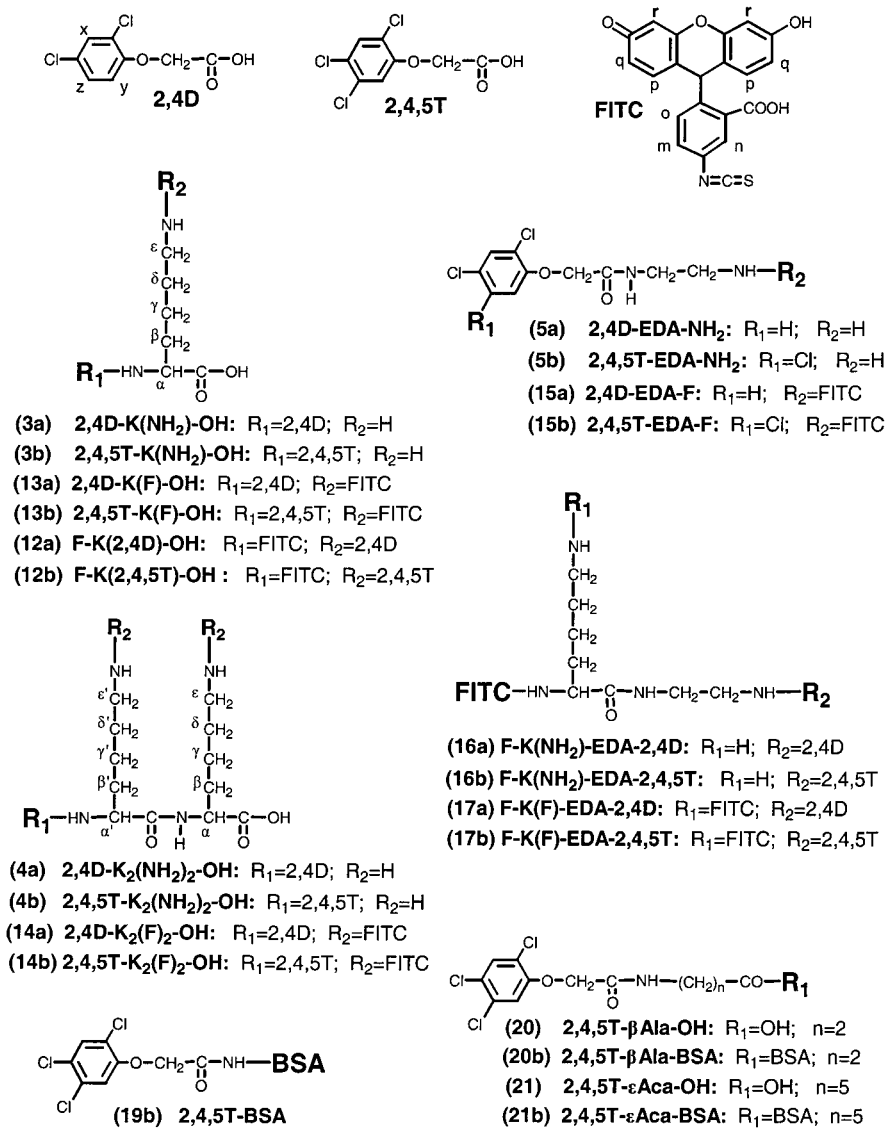

Figure 1. Chemical structures of synthesized derivatives, tracers, and immunogens. For each compound a code number (in parentheses) and a short name (with **boldface** letters) are given. The short name has been chosen to describe the corresponding chemical structure. When an **R** group is noted as 2,4D or 2,4,5T, it represents their acyl derivatives. When **R** is given as FITC, it represents the thiocarbamoyl fluorescein. The notation with Greek characters in L-lysine and with Latin characters in 2,4D and FITC is used for the study of the ^{1}H NMR chemical shifts and for the study of the Figures S1−S4 of the Supporting Information. Replacing the two chlorine atoms from **15a** with two bromine atoms can confirm the structure of tracer **15c**.

was 284 nm, and the UV spectrum was identical to that of 2,4D, UV_{max} of **3b** and **4b** was 289 nm, and UV spectrum was identical to that of 2,4,5T. Derivatives **3a** and **4a** were further characterized with NMR spectroscopy. ***3a*** *^{1}H NMR (MeOH-d_4):* δ = 1.39 (m, 2H, CH_2-γ), 1.65 (m, 2H, CH_2-δ), 1.83 (td, 2H, CH_2-β), 2.87 (t, 2H, CH_2-ϵ), 4.35 (t, 1H, CH-α), 4.59 (s, 2H, OCH_2CO), 7.05 (d, 1H, H-y), 7.25 (dd, 1H, H-z), 7.42 (d, 1H H-x). ***4a*** *^{1}H NMR (MeOH-d_4):* δ = 1.44 (m, 4H, CH_2-γ,γ'), 1.64 (m, 4H, CH_2-δ,δ'), 1.75 (td, 2H, CH_2-β), 1.81 (td, 2H, CH_2-β'), 2.86 (m, 4H, CH_2-ϵ,ϵ'), 4.28 (m, 1H, CH-α), 4.43 (t, 1H, CH-α′) 4.61 (s, 2H, OCH_2CO), 7.02 (d, 1H, H-y), 7.25 (dd, 1H, H-z), 7.42 (d, 1H H-x). The notation of the protons is given in Figure 1.

*2,4D-EDA-NH_2 (**5a**), 2,4,5T-EDA-NH_2 (**5b**), and 2,4DBr-EDA-NH_2 (**5c**).* A 10 mL volume of DMF and 1 mL of EDA were added in 1 g of CLTR under agitation and left 30 min for EDA immobilization on resin. The resulting resin (H_2N-EDA-CLTR) washed as usually and dried. A 0.45 mmol amount of each analyte (2,4D, 2,4,5T, and 2,4DBr) was used for reaction with 100 mg of H_2N-EDA-CLTR and the condensing agent NHS/DCC. Haptens 2,4D-EDA-NH_2 (**5a**), 2,4,5T-EDA-NH_2 (**5b**) (Figure 1), and 2,4DBr-EDA-NH_2 (**5c**) were isolated as above. All derivatives exhibited a positive ninhydrin test with R_f**5a** = 0.39 and R_f**5b** = 0.42. The UV spectra of **5a,b** were identical to those of 2,4D and 2,4,5T, respectively.

*H_2N-K(Mtt)-EDA-2,4D (**6a**) and H_2N-K(Mtt)-EDA-2,4,5T (**6b**).* Equal molar quantities of Fmoc-K(Mtt)-OH and the derivatives **5a,b** were used for a 24 h reaction using DCC/HOBt as the condensing agent. The products were treated for 30 min with 25% isopropylamine in dichloromethane (DCM) for the Fmoc removal, and the derivatives H_2N-K(Mtt)-EDA-2,4D (**6a**) and H_2N-K(Mtt)-EDA-2,4,5T (**6b**) were isolated with diethyl ether precipitation and preparative TLC. Spots with R_f**6a** = 0.43 and R_f**6b** = 0.47 which give a positive ninhydrin test were collected and products eluted with methanol.

*H_2N-K(NH_2)-EDA-2,4D (**7a**) and H_2N-K(NH_2)-EDA-2,4,5T (**7b**)*. Derivatives **7a,b** were easily prepared as described in the previous paragraph using the L-lysine derivative Fmoc-K(Fmoc)-OH instead of Fmoc-K(Mtt)-OH. With this modification two amino groups were incorporated in the same molecule. Spots with R_f**7a** = 0.04 and R_f**7b** = 0.06 were collected from preparative TLC. UV spectra of **7a,b** were identical to those of 2,4D and 2,4,5T, respectively.

*F-K(2,4D)-OH (**12a**) and F-K(2,4,5T)-OH (**12b**)*. The protected L-lysine derivative Fmoc-K(Mtt)-OH (0.3 mmol) was loaded on 500 mg of CLTR as described above. Fmoc was removed, and the resulting resin washed and dried as usually. The free α-amino group reacted with FITC. Briefly, 4.7 mg of FITC dissolved in 0.5 mL of DMF was mixed with 20 mg of resin (H_2N-K(Mtt)-CLTR) and allowed for react for 1 h . The usual washes followed, and the fluorescent product was cleaved from resin and Mtt deprotected, with 1% TFA solution in DCM for 20 min. The filtrate evaporated, and the fluorescent derivative F-K(NH_2)-OH was isolated with ether precipitation, ether wash, and drying (3.5 mg). A 0.0065 mmol amount from each 2,4D and 2,4,5T was activated with the condensing agent NHS/DCC (1 mg/1.5 mg) in 70 μL of DMF and reacted in two separate reaction vessels with 0.004 mmol of the F-K(NH_2)-OH derivative for 24 h. DMF was evaporated under vacuum, and 2 mL of diethyl ether was added in each tube for precipitation of tracers. The tracers F-K(2,4D)-OH (**12a**) and F-K(2,4,5T)-OH (**12b**) (Figure 1) were washed with ether and dried. They were purified with preparative TLC (R_f**12a** = 0.4 and R_f**12b** = 0.42) and stored in methanolic solution.

*Preparation of Fluorescent Tracers **13a**,**b**, **14a**,**b**, **15a**−**c**, **16a**,**b**, and **17a**,**b***. The derivatives **3a,b**, **4a,b**, **5a**−c, **6a,b**, and **7a,b** were dissolved in MeOH and reacted with FITC.[29] The molar ratio used in reaction was FITC:amino groups = 1.5:1. For 0.06 mmol of amino groups, 0.5 mL of MeOH and 5 μL of diisopropyl ethylamine (DIPEA) were used. After the completion of reaction, solvent was evaporated and tracers were easily precipitated with diethyl ether. The precipitates were washed with ether and purified with preparative TLC. In the case of the **6a,b** derivatives, the Mtt moiety was removed just after the reaction with FITC, with 1% TFA in DCM for 20 min. R_f**13a** = 0.35, R_f**13b** = 0.38, R_f**15a** = 0.52, R_f**15b** = 0.53, R_f**16a** = 0.15, R_f**16b** = 0.16, R_f**14a** = 0.18, R_f**14b** = 0.2, R_f**17a** = 0.23, and R_f**17b** = 0.25. All tracers exhibited UV_{max} = 492 nm in a 0.1 M $NaHCO_3$ solution, and spectra were identical to that of fluorescein. The ninhydrin test in all cases was negative except in tracers **16a,b**. The structure of tracers **13a** and **14a** was confirmed with NMR spectroscopy. ***13a*** *^{1}H NMR (MeOH-d_4):* δ = 1.40 (m, 2H, CH_2-γ), 1.64 (m, 2H, CH_2-δ), 1.84 (td, 2H, CH_2-β), 3.56 (b, 2H, $CH_{2\epsilon}$), 4.34 (b, 1H, CH-α), 4.58 (s, 2H, OCH_2CO), 6.51 (dd, 2H, H-q), 6.64 (d, 2H, H-r), 6.71 (d, 2H, H-p), 7.02 (d, 1H, H-y), 7.08 (d, 1H, H-o), 7.23 (dd, 1H, H-z), 7.38 (d, 1H H-x), 7.70 (d, 1H, H-m), 8.07 (s, 1H, H-n). ***14a*** *^{1}H NMR (MeOH-d_4):* δ = 1.46 (m, 4H, CH_2-γ,γ′), 1.62 (m, 4H, CH_2-δ,δ′), 1.81 (m, 2H, CH_2-β), 1.86 (m, 2H, CH_2-β′), 3.59 (m, 4H, CH_2-ϵ,ϵ′), 4.32 (t, 1H, CH-α), 4.48 (t, 1H, CH-α′) 4.62 (s, 2H, OCH_2CO)), 6.53 (dd, 4H, H-q), 6.62 (d, 4H, H-r), 6.78 (m, 4H, H-p), 6.96 (d, 1H, H-y), 7.05 (m, 2H, H-o), 7.19 (dd, 1H, H-z), 7.33 (d, 1H H-x), 7.70 (d, 2H, H-m), 8.07 (s, 2H, H-n). The structures of all tracers and notations of the protons are given in Figure 1.

Table 1. Results from 2,4D FPIA, Obtained Using All Synthesized Tracers and the E2/G2 Monoclonal Antibody: Comparison of Sensitivities

tracer	concn (nM)[a]	I_{50} (ng/mL)[b]	ratio[c]
2,4DK(F)-OH (**13a**)	2	12.2	1
2,4D-EDA-F (**15a**)	2	65	5.3
2,4DBr-EDA-F (**15c**)	2	153.8	12.6
FK(2,4D)-OH (**12a**)	2	93.5	7.6
FK(NH2)-EDA-2,4D (**16a**)	2	111.9	9.17
2,4DK2(F)2OH (**14a**)	8	87.8	7.2
FK(F)-EDA-2,4D (**17a**)	4	102.7	8.4

[a] Final concentration in reaction mixture. [b] I_{50}: concentration of 2,4D that reduces the initial polarization (mP_0) at 50%. [c] Ratio: I_{50} of a tracer/I_{50} of **13a**. This ratio is an index of the relative sensitivity between tracers.

*2,4,5T Haptens 2,4,5T-βAla-OH (**20**) and 2,4,5T-ϵAca-OH (**21**)*. Two portions of 200 mg of CLTR each were loaded with Fmoc-βAla-OH (0.2 mmol) and Fmoc-ϵAca-OH (0.2 mmol) and treated as described above. Resins H_2N-βAla-CLTR and H_2N-ϵAca-CLTR had a substitution of ∼0.5 mmol/g. Herbicide 2,4,5T reacted in a 5 molar excess with both resins as already described. Haptens were isolated after cleavage with 90% TFA in water. The TFA was evaporated, and the products were precipitated with water, washed two times, and dried under vacuum. Derivatives **20** and **21** (Figure 1) exhibited UV_{max} = 289 nm and spectra identical to that of 2,4,5T.

*Synthesis and Molar Ratio Calculation of 2,4,5T Immunogens 2,4,5T-BSA (**19b**), 2,4,5T-βAla-BSA (**20b**), and 2,4,5T-ϵAca-BSA (**21b**)*. Immunogen syntheses were performed with a modified carbodiimide method. Briefly, 7.65×10^{-3} mmol of each hapten was activated with 0.01 mmol of NHS and 0.01 mmol of DCC in 100 μL of DMSO. After DCU precipitation the supernatants were added to a 1.2 mL solution of 14 mg of BSA in DMSO/carbonate buffer (0.05 M; 1:1). After 24 h of reaction, the solutions were dialyzed against water and stored at −20 °C. The molar ratio calculation of the immunogens (Figure 1) was performed according to Franek.[18] The 2,4,5T-BSA (**19b**) molar ratio = 20:1, 2,4,-5T-βAla-BSA (**20b**) molar ratio = 13.5:1, and 2,4,5T-ϵAca-BSA (**21b**) molar ratio = 24/1.

Immunization and Antiserum Production. Immunization of six (two for each immunogen) New Zealand rabbits were carried out by intradermal administration of immunogens with Freund's complete adjuvant as elsewhere described.[30] Blood samples were collected 10 days after the boosts. The antiserum was fractionated with 40% saturated ammonium sulfate solution and the pellet washed twice with 40% saturated ammonium sulfate and stored as suspension at 4 °C until use.

FPIA Procedures. All dilutions of tracers and antibodies were performed in TDx Abbot buffer. Tracer titer was chosen as the tracer concentration with a fluorescence intensity of approximately 1000. Final concentrations of all 2,4D tracers used are listed in Table 1.

Eight serial dilutions for each antibody were prepared, starting from 1:200. Antibodies dilution curves were obtained by adding

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250 μL of tracer (titer dilution), 150 μL of each of the antibody dilutions, and 300 μL of TDx buffer. The polarization was measured just after mixing of the reagents. The antibody dilution with a polarization 30−40% below the maximum value was chosen as the antibody titer.[8]

Standard solutions of 2,4D and 2,4,5T were prepared from methanolic stock solutions (1 mg/mL) in bottled water with concentrations 0.1, 1.0, 10, 100, 1000, and 10000 ng/mL.

Standard curves were obtained using 250 μL of tracer (titer dilution), 150 μL of antibody (titer dilution), 100 μL of standard solution of analyte, and 200 μL of TDx buffer. The polarization measurements were performed after 5 min of incubation at RT (room temperature).[8] The I_{50} values were calculated using the Microcal Origin software with sigmoidal fitting.

Cross- reactivity studies were performed with the most closely structurally related compounds to evaluate the influence of new tracers on the specificity of FPIA.

Dissociation constants were calculated with Schatchard plot analysis according to Dadlinker[31] using polarization data.

Apparatus. Fluorescence intensity and polarization measurements were performed with the TDx Abbot fluorometer in photocheck mode. 1D (^{1}H, ^{13}C) and 2D (gs-COSY, gs-HMQC, gs-HMBC) NMR spectra were obtained on a Bruker AMX-500 spectrometer using MeOH-d_4 as a solvent and at a temperature of 25 °C. Chemical shifts reported are in ppm from internal TMS.

RESULTS AND DISCUSSION

Tracer Selection and Synthesis. For the preparation of immunogen molecules and subsequent generation of antibodies, the appropriate hapten molecules are usually attached onto the surface of a carrier protein through a four-carbon aliphatic side chain of L-lysine molecules. Antibodies against such immunogens often recognize conjugated analytes better than free analytes due to bridge recognition by the antibody.[6,19] This leads to low sensitivity, as competition between the free and conjugated analyte (tracer) is favored for the tracer. To address this problem in terms of FPIA, we prepared a series of 2,4D and 2,4,5T tracers (**12a,b**, **13a,b**, **16a,b**) using L-lysine-based derivatives which we considered as homologous or heterologous to immunogen structure (Figures 1 and 2). Tracers with two fluorescent molecules (bilabeled) were also prepared (**14a,b**, **17a,b**) to examine the potential use of multilabeled tracers and the structure dependency of quenching in FPIA. Gerdes et al. have used closely related cross-reactants instead of 2,4D in ELISA conjugates to enhance competition.[32,33] To address this, we prepared tracer **15c** (2,4DBr-EDA-F) on the basis of the cross-reactant 2,4DBr. In addition the EDA-based tracers 2,4D-EDA-F (**15a**) and 2,4,5T-EDA-F (**15b**) were prepared for comparison purposes, as these tracers were considered by other researchers as the most appropriate for FPIA development.[8,16,21]

In Figure 1 the chemical structures of synthesized derivatives and FITC tracers are shown. The solid-phase synthetic strategy was favored because it provided advantages in synthesis and isolation of high-purity haptens, derivatives, and tracers with satisfactory reaction efficiencies. The introduction of L-lysine allowed us to synthesize a variety of monolabeled and bilabeled tracers. All derivatives exhibited a single spot in the TLC, their UV spectrum was identical with that of the corresponding analyte, and the ninhydrin test was positive. The structures of **3a** and **4a** that were considered as the most important derivatives were confirmed with ^{1}H NMR spectroscopy, the chemical shifts of which are given in the Experimental Section. The assignment of their ^{1}H NMR spectra was performed using ^{1}H−^{1}H gs-COSY, ^{1}H−^{13}C gs-HMQC, and ^{1}H−^{13}C gs-HMBC 2D NMR techniques (Figures S1, S2, and S4 in the Supporting Information).

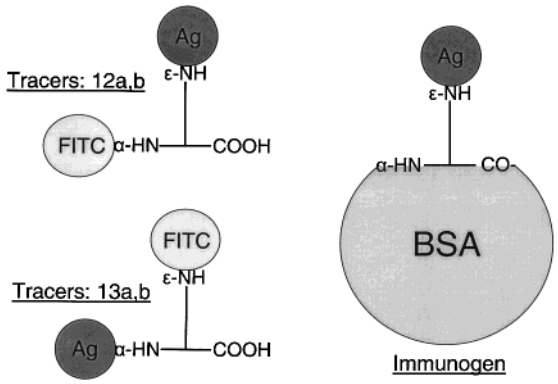

Figure 2. Schematic representation of the structural homology appearing between immunogen and tracers **12a,b** and the structural heterology between immunogen and tracers **13a,b**. Immunogen and tracers **12a,b** have the antigen (2,4D or 2,4,5T) attached to the amino group of the ϵ-primary aliphatic carbon atom of L-lysine. Tracers **13a,b** have the antigen attached to the aminogroup of the α-secondary asymmetric carbon atom of L-lysine.

The very well known final reaction of all amino group containing derivatives with FITC[29] yielded, in all cases, crude preparations with a main spot in the TLC corresponding to the expected tracer as confirmed with polarization change measurements using the appropriate antibody. All tracers were purified with preparative TLC. The structure of tracers **13a** and **14a** was confirmed with ^{1}H and ^{13}C 1D and 2D NMR spectroscopy. The ^{1}H NMR chemical shifts of **13a** and **14a** are given in the Experimental Section. In the Supporting Information (Figure S3) the ^{1}H−^{1}H gs-COSY 2D NMR of **13a** tracer is given.

Concentration of Tracers: Influence of Quenching. The concentration of all fluorescent tracers was determined using $\epsilon_{492} = 68$ mM^{-1} cm^{-1} for conjugated FITC.[34] All monolabeled tracers (**12a**, **13a**, **15a**, **16a**) exhibited approximately 50% lower fluorescence intensity (FI) when compared with the same concentration of free fluorescein, due to quenching caused by hapten-to-dye interaction[13] (Figure S5 in the Supporting Information). The working concentration (titer) of these tracers in FPIA was set at 2 nM with fluorescence intensity about 1000. In bilabeled tracers an additional quenching due to dye-to-dye interaction was observed, further reducing the quantum yield. We assumed that this quenching was structure dependent because tracer **14a** exhibited even lower FI than tracer **17a**. This could be explained taking into account the high flexibility of the two side chains of the connected lysine moieties and the consequent high possibility of

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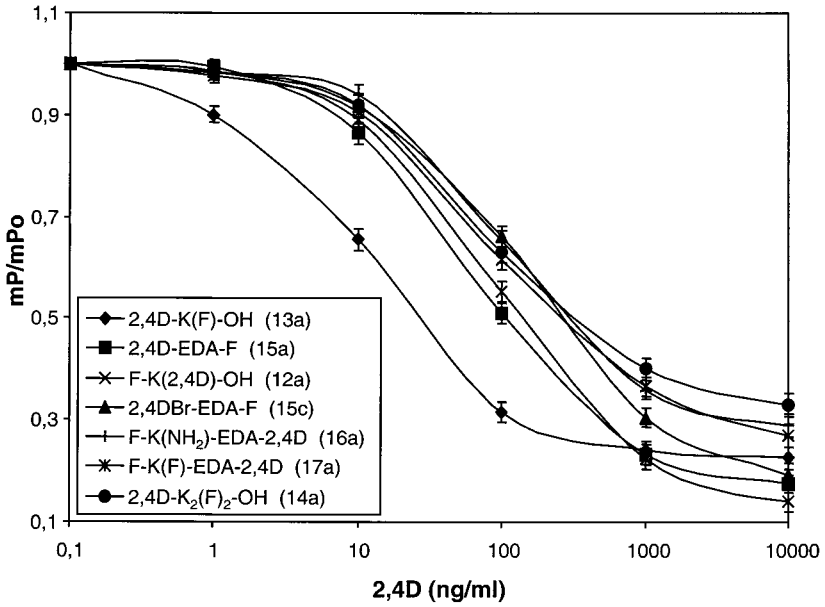

Figure 3. Standard curves obtained using the monoclonal antibody E2/G2 and the 2,4D tracers **13a**, **15a**, **12a**, **15c**, **16a**, **17a**, and **14a**. In all cases, mP_o was approximately 150. Each standard curve was the mean of four curves obtained from separate experiments. The error bars presented in the graphs are ±1 SD.

interaction between the two fluorescein molecules, as compared with the interaction between the α- and ϵ-fluoresceins found in tracer **17a** (Figure 1). The bilabeled tracers **14a** and **17a** were used in FPIA with concentrations 8 and 4 nM, respectively. A tracer with six fluorescein molecules (2,4D-$K_6(F)_6$-OH) was also prepared, which exhibited negligible FI when compared with the other tracers showing the high degree of dye-to-dye quenching when the number of dyes/tracer molecule increases (Figure S5 in the Supporting Information).

2,4D Assay. *Standard Curves and Determination of Sensitivities.* For 2,4D FPIA, we used the monoclonal antibody E2/G2. It has been also used previously in 2,4D immunoassays.[1,24,33,35] In combination with the 2,4D-EDA-F (**15a**) tracer, equilibrium and kinetic FPIAs have been developed.[8,16] The I_{50} was reported as 100 ng/mL for the equilibrium,[8] and the LOD, as 4 ng/mL for the kinetic FPIA.[16] To compare tracers, the I_{50} values indicative of sensitivity were calculated and presented in Table 1. The new L-lysine-based tracer 2,4D-K(F)-OH (**13a**) exhibited $I_{50} = 12.2$ ng/mL and was far more sensitive than all other tracers. The isomeric tracer F-K(2,4D)-OH (12a) exhibited $I_{50} = 93.5$ ng/mL, 7.6 times higher. Tracer **13a** was also 5.3 times more sensitive than the EDA-based one (**15a**), which exhibited $I_{50} = 65$ ng/mL. The high I_{50} obtained using tracer **12a** can easily be explained by taking into account its total homology with immunogen as Figure 2 shows. 2,4D is attached via the amino group to the ϵ-primary aliphatic carbon of L-lysine, in both the immunogen and the tracer **12a**. In tracer **15a**, 2,4D has been also attached via the amino group to the primary aliphatic carbon of EDA. This chemical similarity in the site of attachment between the two tracers is probably the reason the high I_{50} also obtained with tracer **15a**. In the case of tracer **13a**, 2,4D is attached via the amino group to the α-secondary carbon atom of L-lysine. This carbon is additionally asymmetric and brings a carboxyl group. In our point of view these structural differences in the site of attachment lead to more specific recognition of the tracer by the antibody and limited bridge recognition, and a significant improvement in 2,4D FPIA sensitivity is reached ($I_{50} = 12.2$ ng/mL). Tracer **13a** was also more sensitive than the bilabeled tracers **14a** and **17a**, the sensitivities of which were limited by their high concentration in reaction mixture due to quenching (Table 1). From comparison of the bilabeled tracers **14a** and **17a**, we have found that **14a** exhibits better I_{50}, while this is used in double concentration (8 nM) due to strong dye-to-dye quenching. Tracer **14a** is a tether structure bilabeled tracer, chemically similar to **13a** (Figure 1). This structural similarity is probably the reason for the better sensitivity obtained when **14a** compares to **17a**, which is chemically similar with **15a**. Tracers such as **14a** and generally multilabeled tethers can be of great value in assays, which are not affected by quenching, or in FPIAs with resistant to quenching dyes. Antigen heterologous tracer **15c**, with the 2,4DBr cross reactant, exhibited surprisingly high I_{50} (~154 ng/mL) suggesting that important bridge recognition was responsible for the low sensitivity. Additionally, the weaker recognition of 2,4DBr from the antibody led to a reduced mP_O value. The antibody concentration was increased 1.5 times in reaction mixture, and an adequate mP_O value (150) was reached. This critical increase in antibody concentration could be partially responsible for the high I_{50} value obtained. This finding also suggests that antigen heterologous tracers are not probably suitable for FPIA development.

The standard curves with all tracers and antibody E2/G2 are presented in Figure 3. The improvement in sensitivity obtained with tracer **13a** is clearly demonstrated. The dynamic range of this curve was 1−100 ng/mL, almost 1 order magnitude better than the other standard curves. The obtained detection limit was 0.8 ng/mL, calculated three SD below the mP_O value.

Measurement of Dissociation Constant (K_d) Values. To explain the difference in sensitivity, the K_d values of E2/G2 antibody with

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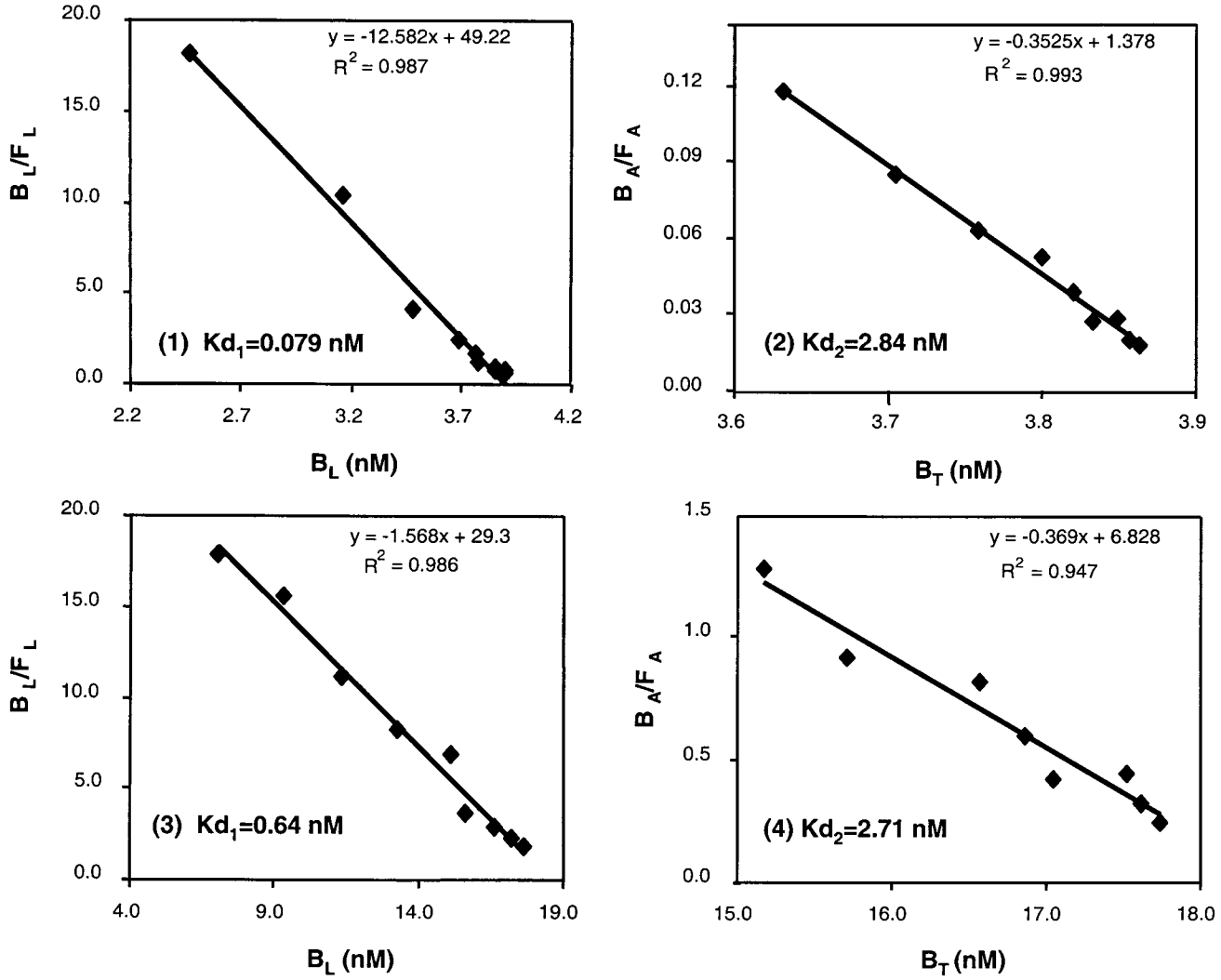

Figure 4. Scatchard plots from experiments "A" (graphs 1 and 2) and "C" (graphs 3 and 4). The equations given in graphs 1 and 3 correspond to the scatchard equation $B_L/F_L = -(1/K_{d1})B_L + R_O/K_{d1}$ for determination of the K_{d1} values, when 2,4D is not present in the reaction mixture ($y = B_L/F_L$, $x = B_L$, and R_O = total antibody concentration). The equations given in graphs 2 and 4 correspond to the scatchard equation $B_A/F_A = -(1/K_{d2})B_T + R_O/K_{d2}$ for determination of the K_{d2} value ($y = B_A/F_A$, $x = B_T$, and R_O = total antibody concentration).

tracers **15a** and **13a** and with 2,4D were determined in solution according to Dadlinker et al.[31] When 2,4D competes the tracer, two parallel equilibrium reactions are taking place with different antibody affinities toward the fluorescent ligand (L) and unlabeled antigen (A):

$$R + L \rightleftarrows R{-}L \quad K_{d1}$$

$$R + A \rightleftarrows R{-}A \quad K_{d2}$$

R is antibody, L is tracer **15a** or **13a**, and A is 2,4D. From Scatchard plots of B_L/F_L versus B_L when unlabeled antigen (2,4D) is not present in solution and B_A/F_A versus B_T in the presence of 2,4D, K_{d1} and K_{d2} values were respectively calculated. *B* and *F* are the bound and free fractions, respectively, and B_T is the total bound fraction ($B_T = B_L + B_A$). K_{d2} should be independent of the tracer used, because it represents the antibody−2,4D dissociation constant. The difference in tracer structure, the degree of bridge recognition by the antibody, and the homology or heterology should lead in tracer-dependent K_{d1}. Usually the antibody has higher affinity for the tracer than the free analyte. A sensitive assay is obtained when the affinity between the tracer and the analyte is comparable.[6] For each tracer two independent experiments with different antibody quantities each were executed in duplicate. A and B experiments were performed with tracer **15a**, and C and D experiments with tracer **13a**. The results from Scatchard plots are summarized in Table 2. The Scatchard graphs from experiments A (graphs 1 and 2) and C (graphs 3 and 4) are shown in Figure 4. In all experiments, the K_{d2} values were calculated and found as expected practically stable, representing the affinity between the antibody and the free analyte (2,4D). From K_d ratios, we assumed that the antibody affinity for tracer **15a** was much higher (35 times) than that of free 2,4D. On the contrary, the affinity for tracer **13a** was only 4.3 times higher explaining the difference in sensitivity (I_{50}) observed between the two tracers. The ratio in association constants [$1/K_{d1}$(**15a**):$1/K_{d1}$(**13a**)] of the two tracers was 8.1. This difference in affinity can be explained accepting that there is important bridge recognition in the case of tracer **15a** and negligible bridge recognition for tracer **13a**, explaining its specific nature. The competition of 2,4D was much

Table 2. Dissociation Constants and Relative Affinities of Tracers 15a and 13a (K_{d1} Values) and Herbicide 2,4D (K_{d2} values) with the E2/G2 Monoclonal Antibody

	2,4D-EDA-F (**15a**)		2,4DK(F)-OH (**13a**)	
constant[a]	expt A[b]	expt B[b]	expt C[b]	expt D[b]
K_{d1} (nM)	0.079	0.08	0.64	0.65
K_{d2} (nM)	2.84	2.79	2.71	2.83
$1/K_{d1}$:$1/K_{d2}$	35[c]		4.3[d]	
$1/K_{d1}$(**15a**):$1/K_{d1}$(**13a**)[e]	8.1			

[a] $1/K_d$ is the affinity constant. [b] Results from two independent experiments (A, B and C, D) for each tracer. [c] Ratio of the mean values of the affinity constants from experiments A and B. [d] Ratio of the mean values of the affinity constants from experiments C and D. [e] Ratio of the affinity constants between the two tracers.

Table 3. % Cross-Reactivities (% CR) of E2/G2 Antibody with 2,4D Structurally Related Compounds, Using the Tracers 2,4D-K(F)-OH (**13a**) and 2,4D-EDA-F (**15a**)[a]

cross-reactant	% CR with 2,4D-K(F)-OH	% CR with 2,4D-EDA-F
2,4D	100	100
2,4,5T	3.2	3.8
(2,4-dibromophenoxy)acetic acid (2,4DBr)	59.8	60.3
2,4-dichlorophenol (2,4D-OH)	1.4	1.4
(2,4-dimethylphenoxy)acetic acid (2,4DMPA)	1.9	1.8
(2,5-dichlorophenoxy)acetic acid (2,5D)	1.7	2.5
(2-chlorophenoxy)acetic acid (2CPA)	1.0	1.2
(4-chlorophenoxy)acetic acid (4CPA)	0.5	0.6
(2-chloro-4-fluorophenoxy)acetic acid (2C 4F)	1.2	6.5
(2,4-dichlorophenoxy)butyric acid (2,4DB)	3.2	3.2
(2,4-dichlorophenoxy)propionic acid (2,4DP)	<0.1	<0.1
2,4-D methyl ester (2,4D-OMe)	220.3	201.9

[a] Cross-reactivities have been calculated using the equation % CR = (I_{50} of 2,4D/I_{50} of cross-reactant) × 100.

more effective in the case of tracer **13a** giving thus five times better I_{50} and consequently better sensitivity in the assay. The K_d value of 2,4D with E2/G2 has been measured using an immunosensor[24] with absolutely comparable results.

In Table 3 the percent cross-reactivities with the most structurally related compounds are listed. As it can be seen, cross-reactivities when using tracer **13a** were generally similar and in some cases lower when compared with the ones calculated with **15a** tracer. The other characteristics of the assay like recovery, reproducibility, and accuracy are not practically affected and remain as previously reported[8] using the 2,4D-EDA-F tracer.

With the use of the absolutely new L-lysine-based tracer **13a**, main characteristics of 2,4D assay have been significantly improved (sensitivity, detection limit) or remained unaffected (specificity, reproducibility), suggesting the use of this tracer for enhancing the performance of 2,4D FPIA.

2,4,5T Assay. From the above results some questions were raised having to do with structure **13** (Ag-K(F)-OH). (a) Is this a universal structure that could be adapted and improve other FPIAs? (b) Can the same results be obtained with polyclonal antibodies also? (c) Which is the most compatible immunogen for this structure?

The herbicide 2,4,5T was used to prepare haptens and immunogens as already described in the Experimental Section.

Table 4. I_{50} Values and Their Ratio Obtained from the 3rd and 4th Bleeding of All Immunized Rabbits, Using the Tracers 13b and 15b

antiserum	2,4,5T-K(F)-OH (**13b**) I_{50} (ng/mL)	2,4,5T-EDA-F (**15b**) I_{50} (ng/mL)	I_{50}(**15b**)/I_{50}(**13b**)[a] ratio
R1 3rd bleeding	226	1028	4.5
R2 3rd bleeding	133	701	5.3
R3 3rd bleeding	217	1180	5.4
R4 3rd bleeding	85	761	8.9
R5 3rd bleeding	674	2750	4.1
R6 3rd bleeding	nd	4517	
R1 4th bleeding	151	793	5.3
R2 4th bleeding	212	940	4.4
R3 4th bleeding	212	1710	8.1
R4 4th bleeding	91	845	9.3
R5 4th bleeding	385	1800	4.7
R6 4th bleeding	nd	3786	
a-2,4,5T(UK)	389	1877	4.8

[a] This is an index of relative sensitivity between the two tracers indicating the constant improvement obtained with tracer **13b**.

Three haptens were synthesized, using solid-phase chemistry, differing in bridge length: 2,4,5T-BSA (**19b**), 2,4,5T-βAla-BSA (**20b**), and 2,4,5T-ϵAca-BSA (**21b**) (Figure 1). With these immunogens six rabbits were immunized, rabbits 1 and 2 with **19b**, rabbits 3 and 4 with **20b**, and rabbits 5 and 6 with **21b**. Antiserums after the third bleeding were collected and evaluated for sensitivity with the corresponding 2,4,5T tracers.

The antiserum from rabbit 3, 3rd bleeding, which exhibited high titer (1/3000), was initially used to evaluate all different 2,4,-5T tracers. As expected from 2,4D experience, tracers 2,4,5T-K(F)-OH (**13b**) and 2,4,5T-EDA-F (**15b**) gave better results, and therefore, they were used to evaluate the ammonium sulfate fractionated antiserums from all immunized rabbits. The resulting I_{50} values and their ratio from 3rd and 4th bleedings are presented in Table 4. Tracer **13b** gives in all cases lower I_{50} values when compared with **15b**. Similar results in ratios were obtained using another polyclonal antibody against 2,4,5T kindly provided by Dr. Ramadan Abuknesha from King's College University of London with the short name a-2,4,5T(UK). The ratio I_{50}(**15b**)/I_{50}(**13b**) varies from 4 to approximately 9. The antiserums from rabbits 3 and 4 corresponding to the **20b** immunogen with β-alanine as spacer exhibited the higher ratios. The most sensitive antiserums were also obtained from rabbit 4, and this was further evaluated. Antiserum from rabbit 4, 3rd bleeding, exhibited remarkable sensitivity especially when used with the **13b** tracer. Standard curves from R4 3rd bleeding antiserum and tracers **13b**, **15b**, and **12b** are provided as Supporting Information (Figure S6). The other tracers including the bilabeled **14b** and **17b** exhibited lower sensitivities due to quenching. The dynamic range of the **13b** tracer curve was from 10 to 1000 ng/mL with I_{50} = 85 ng/mL and detection limit at 5 ng/mL. The standard curves corresponding to the other 2,4,5T tracers appeared with a dynamic range at least 1 order magnitude higher.

The detailed evaluation of the anti 2,4,5T antibodies is beyond the aim of this paper. We were focused mainly on the new L-lysine-based tracer structure performance when used with different analytes (2,4D and 2,4,5T) and with monoclonal or polyclonal antibodies derived from different immunogens. We found that,

in all cases, sensitivity (I_{50}) was improved at least 5 times when the new tracers **13a,b** were used. The ratio in I_{50} values between **15b** and **13b** tracers was increased up to 9 times when antibodies corresponding to immunogen with the three-carbon spacer β-alanine were used.

CONCLUSION

With the use of L-lysine-based tracers **13a,b** we have significantly improved the sensitivity of 2,4D and 2,4,5T FPIAs because the antibody recognition for tracer is specific and the antibody affinity for tracer is comparable with the affinity for the free analyte. From our point of view these tracers could be adapted with success also in other analytes. A number of further improvements including the use of new dyes resistant to quenching, new instruments, and new technologies, as mentioned in the Introduction, combined with the tracers presented here could give FPIAs comparable in sensitivity with that of ELISA. This is of great importance taking into account the unrivalled simplicity of FPIA. Our current and future efforts are (a) to apply these materials for spiked and real sample measurements, like natural water, white wine, and grape juice, (b) to apply to other FPIA types such as stopped-flow FPIA, minimizing matrix effect, and further improving analysis performance, and (c) to apply to a number of other analytes, including pesticides mycotoxins, drugs, and others.

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SUPPORTING INFORMATION AVAILABLE

Figures S1−S6, showing the ^{1}H−^{1}H homonuclear gradient double quantum filtered COSY-DQF 2D NMR spectrum of derivative **3a**, the ^{1}H−^{1}H homonuclear gradient double quantum filtered COSY-DQF 2D NMR spectrum of derivative **4a**, the ^{1}H−^{1}H homonuclear gradient double quantum filtered COSY-DQF 2D NMR spectrum of derivative **13a**, the carbonyl region of the ^{1}H−^{13}C heteronuclear gradient, multiple bond correlation HMBC 2D NMR spectrum of derivative **4a**, the influence of hapten-to-dye and dye-to-dye quenching in 2,4D tracers, and standard curves from R4 3rd bleeding antiserum and tracers **13b**, **15b**, and **12b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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