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# FLUORESCENT DERIVATIVES OF THROMBOXANE $B_2$: SYNTHESIS, SPECTROSCOPIC AND IMMUNOLOGIC PROPERTIES

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**Summary**—Thromboxane $B_2$ has been labeled by four fluorescent probes structurally related to coumarin (4-bromomethyl-7-methoxycoumarin, 7-[(chlorocarbonyl) methoxy]-4-methylcoumarin, 4-luminarin) or anthracen (panacyl bromide). The purity of the derivatives determined by liquid chromatography was over 90%. The extinction coefficient, Stokes' shift, quantum yield, life-time and the anisotropy of the emitted fluorescence were determined. Immunorecognition of thromboxane $B_2$ derivatives was checked by competition immunoassays. Among the derivatives tested, that obtained with 4-luminarin has a suitable Stokes' shift (95 nm), a quantum yield of 0.46, a single value of excited state life-time (9.3 nsec), a well-preserved immunorecognition and a good chemical stability. Preliminary results in competition experiments showed variations in fluorescence anisotropy correlated to thromboxane $B_2$ concentration.

The identification and quantification of prostaglandins and thromboxanes have been investigated in numerous ways. A variety of analytical methods, such as liquid chromatography,[1-4] gas chromatography combined with mass spectrometry,[5-7] radioimmunoassays (RIA)[8] and non-isotopic immunoassays[9,10] have been used for the determination of eicosanoids. However, all these methods require specific and expensive instrumentation or are relatively time-consuming. Considerable interest has been shown in recent years in homogenous fluoroimmunoassays. Fluorescence polarization immunoassays (FPIA) are based on a change in the anisotropy of the emitted fluorescence by a probe linked to an antigen or an antibody when the antigen–antibody complex is formed. They have been developed for a large variety of antigens.[11-14]

FPIA previously reported generally involve the use of fluorescein or fluorescein derivatives as the fluorescent probe. The presence of contaminants in the commercial preparations,[15] the variations of the fluorescence intensity with pH, its vulnerability to scattered-light interference due to its small Stokes' shift and non-commercially available fluorescein reagent to derivatize carboxylic compounds have led us to consider alternative fluorophores.

We derivatized the alcoholic groups of thromboxane $B_2$ ($TxB_2$) by 7-[(chlorocarbonyl) methoxy]-4 methylcoumarin (7-CMMC) whereas the carboxylic function was derivatized either by esterification with 4-bromomethyl-7-methoxycoumarin (BrMmc) or panacyl bromide (BrP), or amidification with 4-luminarin. The resulting derivatives were purified by semi-preparative liquid chromatography. Spectroscopic properties have been determined and immunocompetition of the derivatives was estimated by immunoassays. Preliminary results showed that 4-Lum-$TxB_2$ could be used as labeled antigen in FPIA.

## EXPERIMENTAL

### *Reagents*

All chemicals and solvents were of analytical reagent grade. $TxB_2$, $TxB_2$ antibodies, 4-luminarin (4-Lum), 1-hydroxybenzotriazole (HOBT) and *N*,*N*-dicyclohexylcarbodiimide (DCC) were purchased from Sigma (Saint

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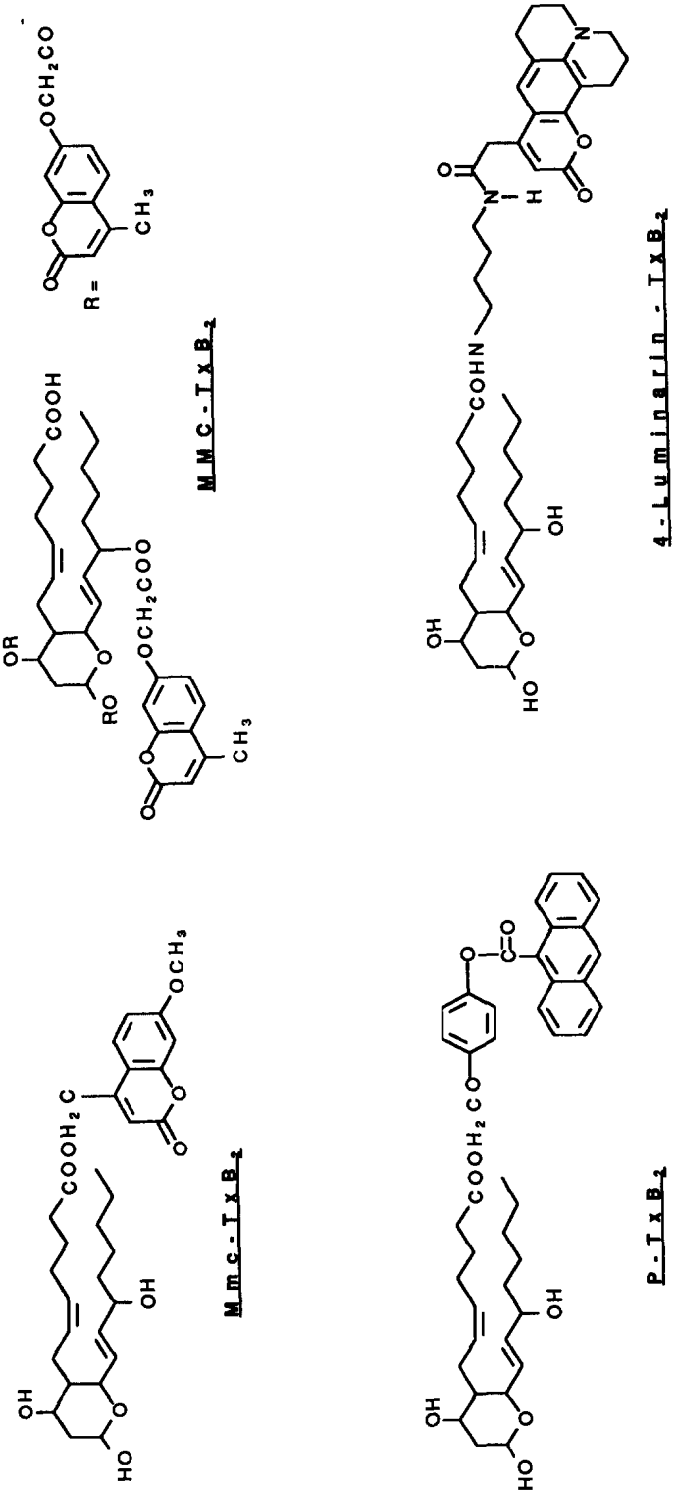


Fig. 1. Chemical structure of the $TxB_2$ derivatives.

Table 1. Conditions selected for esterification of $TxB_2$.

| Reagent | Reagent/$TxB_2$ (Molar ratio) | Temperature | Reaction time |
|---|---|---|---|
| BrMmc | 3 | 70° | 30 min |
| PBr | 3 | 40° | 45 min |
| 7-CMMC | 6 | 60° | 35 min |
| 4-Lum | 3 | Activation step: 20° | 24 hr |
| | | Derivatization: 70° | 2 hr |

Louis, MO, U.S.A.). Panacyl bromide (PBr) was obtained from Molecular Probes (Junction City, OR, U.S.A.), 4-bromomethyl-7-methoxycoumarin (BrMc) and 18-crown-6-ether were supplied by Fluka (Buchs, Switzerland). 7-Hydroxy-4-methylcoumarin, triethylamine, thionyl chloride, 4-dimethylaminopyridine, *N,N*-diisopropylethylamine were obtained from Aldrich (Steims, Germany). *N,N*-dimethylformamide (DMF), dimethylsulfoxyde (DMSO), quinine hydrogenosulphate and propyleneglycol were purchased from Merck (Darmstadt, Germany). 7-[(Chlorocarbonyl) methoxy]-4-methylcoumarin (7-CMMC) was synthesized according to Karlson's method.[16] Acetone and methylene chloride were distilled and dried on a molecular sieve (4 Å).

Immunoassays kits were supplied by various manufacturers: $^3H$ $TxB_2$ RIA kit by NEN (Boston, MA, U.S.A.), $^{125}I$ iodotyrosine-methylester $TxB_2$ RIA kit by Amersham (Les Ulis, France) and the enzymeimmunoassay kit by Cayman (Ann Arbor, MI, U.S.A.).

### *Derivatization procedures*

The reaction time, temperature and fluorescent reagent excess were optimized. $TxB_2$ (1 mg/ml) was dissolved in acetonitrile or in DMF (derivatization with 4-Lum). PBr (0.5 mg/ml) was dissolved in acetonitrile. BrMmc (1.5 mg/ml), 18-crown-6-ether (1 mg/ml) were solubilized in acetone. 7-CMMC (1 mg/ml) and 4-dimethylaminopyridine (1 mg/ml) were dissolved in methylene chloride. DCC (5 mg/ml) and HOBT (5 mg/ml) were solubilized in DMF and 4-Lum (1.5 mg/ml) in DMSO.

*4-Bromomethyl-7-methoxycoumarin derivatization.* BrMmc (15 $\mu$l), 18-crown-6-ether (5 $\mu$l) and potassium carbonate (10 mg) were added to $TxB_2$ (10 $\mu$l). The volume was adjusted to 300 $\mu$l using acetone. The samples were vortex-mixed for 1 min and incubated for 30 min at 70°.

*Panacyl bromide derivatization.* PBr (60 $\mu$l) and triethylamine (10 $\mu$l) were added to $TxB_2$ (10 $\mu$l). The volume was adjusted to 300 $\mu$l using acetone, the samples were vortex-mixed for 1 min and incubated for 30 min at 45°.

*7-[(Chlorocarbonyl) methoxy]-4 methylcoumarin derivatization.* 7-CMMC (45 $\mu$l) and 4-dimethylaminopyridine (20 $\mu$l) were added to $TxB_2$ (10 $\mu$l). The volume was adjusted to 300 $\mu$l using methylene chloride. The samples were vortex-mixed for 1 min and incubated for 35 min at 60°.

*4-Luminarin derivatization.* DCC (10 $\mu$l), HOBT (10 $\mu$l) and triethylamine (10 $\mu$l) were added to $TxB_2$ (10 $\mu$l). The volume was adjusted to 290 $\mu$l using DMF the samples were vortex-mixed for 1 min and incubated while stirring for 24 hr at 20° to obtain the active ester. 4-Lum (10 $\mu$l) was then added. The reaction mixture was left at 70° during 2 hr.

### *Liquid chromatographic systems*

A semi-preparative liquid chromatographic system was used to purify the conjugates. It included a low-pressure pump (Model Duramat, Merck Clevenot), a three-way valve fitted with a 1-ml syringe as injector, a glass column (310 × 25 mm I.D.) packed with LiChroprep Diol (40–63 $\mu$m, Merck) and a UV/visible spectrophotometer (DU 64, Beckman, Gagny, France) equipped with a flow-through cell of 200 $\mu$l and operated at 330 nm for Mmc-$TxB_2$, 254 nm for P-$TxB_2$, 318 nm for MMC-$TxB_2$ and 400 nm for 4-Lum. Mobile phases (hexane-ethanol mixture in various proportions; see Table 2) were used at

Table 2. Chromatographic conditions and capacity factors for each reagent and derivative

| Derivative | Mobile phase (Hexane–Ethanol, v/v) 70–30 | 80–20 | 90–10 |
|---|---|---|---|
| BrMmc | — | — | 1.4 |
| Mmc-$TxB_2$ | — | — | 4.7–8.0 |
| PBr | — | 2.2 | — |
| P-$TxB_2$ | — | 4.0–5.8 | — |
| 7-CMMC | — | — | 3.3 |
| MMC-$TxB_2$ | — | — | 5.4 |
| 4-Lum | 1.7 | — | — |
| 4-Lum-$TxB_2$ | 4.8–6.7 | — | — |

Table 3. Spectroscopic properties of the fluorescent derivatives of $TxB_2$ determined in 0.06*M* phosphate buffer pH 7.3

| | Spectral properties | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Compounds | Ultra Violet $\lambda max$ (*nm*) | $\epsilon$ ($M/l^{-1}$) | $\lambda_{ex}$ (*nm*) | $\lambda_{em}$ (*nm*) | Stokes' shift (*nm*) | Fluorescence $Q_f$ | $\tau$ (*ns*) | $\langle r \rangle$ |
| Mmc-$TxB_2$ | 330 | 12,000 | 330 | 410 | 80 | 0.31 | 3.9 (100%) | 0.048 |
| MMC-$TxB_2$ | 318 | 12,000 | 320 | 380 | 60 | 0.43 | 6.6 (68%)<br>0.6 (32%) | 0.049 |
| P-$TxB_2$ | 375 | 11,000 | 375 | 475 | 100 | 0.20 | 9.8 (70%)<br>2.1 (30%) | 0.240 |
| 4-Lum-$TxB_2$ | 410 | 25,000 | 405 | 500 | 95 | 0.46 | 9.3 (100%) | 0.039 |

$\epsilon$: molar absorptivity.
$Q_f$: quantum yield.
$\tau$: excited state life-time (the numbers between brackets indicate the relative percentage of the populations with different values of life-time).
$\langle r \rangle$: fluorescence anisotropy.

a flow rate of 12 ml/min. The fractions of interest were collected, evaporated to dryness under a gentle stream of nitrogen and their purity was checked on that analytical system. They were then frozen (−20°) before determination of their spectral properties.

The analytical HPLC system consisted of a ternary solvent delivery pump (Model SP 8700, Spectra-Physics, Santa Clara, CA, U.S.A.), an injection valve with a 20 $\mu$l sample loop (Model 7025, Rheodyne, Cotati, CA, U.S.A.) and a fluorescence detector (F1100, Hitachi 655 A-13, Merck-Clevenot, Nogent-sur-Marne, France). The normal phase column prepacked with LiChrospher 100 Diol (LiChrocart 250 × 4 mm I.D., 5 $\mu$m) was purchased from Merck (Damstadt, Germany). The mobile phases (same composition as in semi-preparative HPLC) were filtered through a 0.45-$\mu$m Sartolon microfilter (Sartorius, Göttingen, Germany), degassed by bubbling with helium and used at a flow-rate of 1.2 ml/min. Fluorescence detection was performed at respective excitation and emission wavelengths of 330 and 410 nm for Mmc-$TxB_2$, 254 and 415 nm for P-$TxB_2$, 318 and 378 nm for MMC-$TxB_2$, and 400 and 500 nm for 4-Lum-$TxB_2$. The chromatograms were

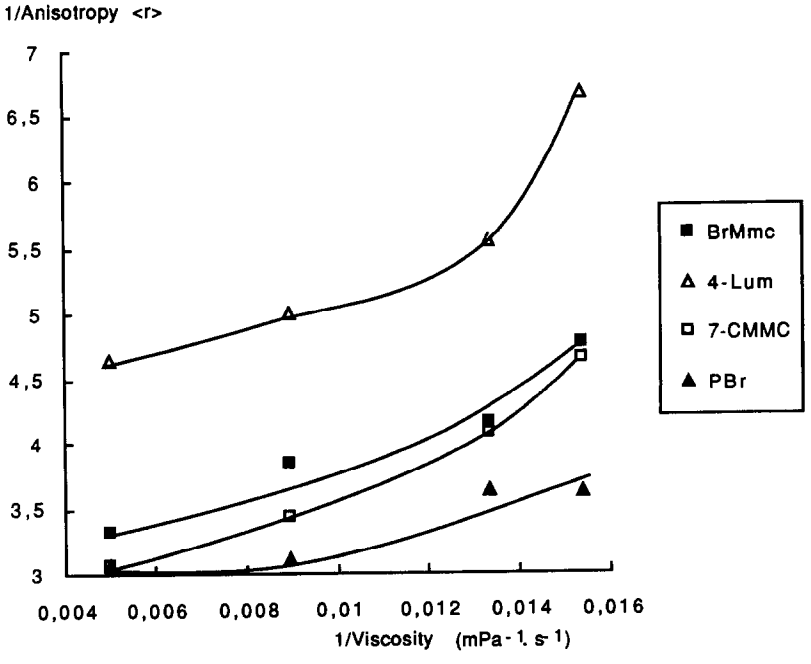


Fig. 2. Fluorescence anisotropy of the fluorescent $TxB_2$ derivatives in relation to viscosity.

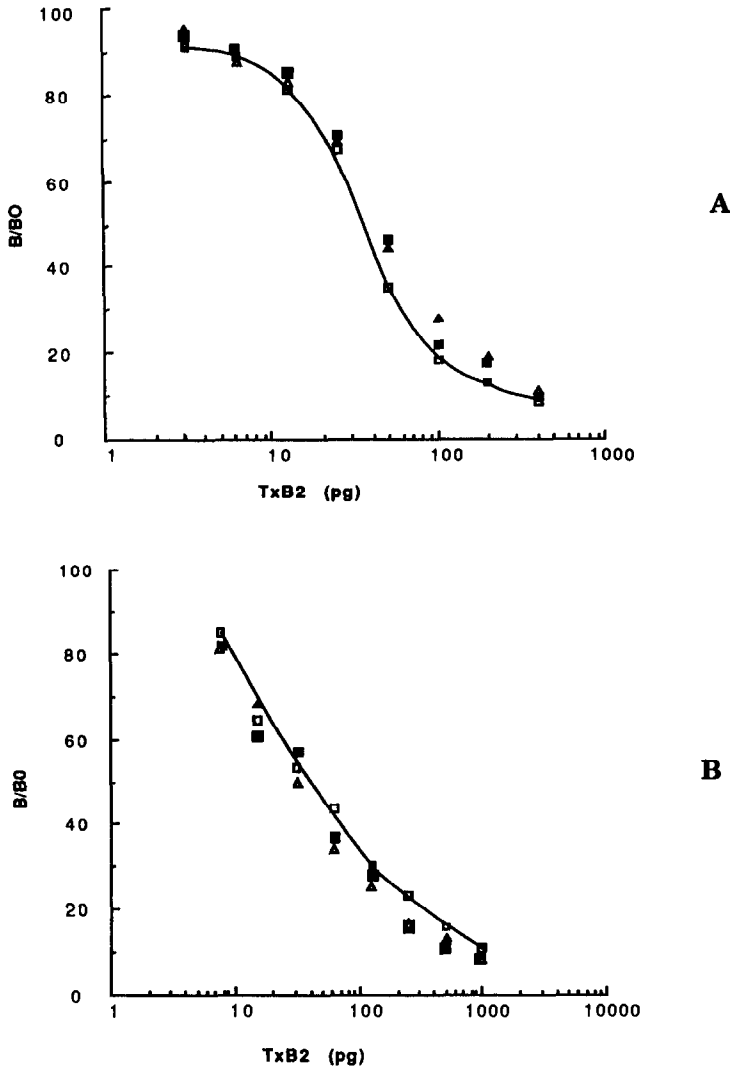


Fig. 3. Dose response curves obtained for $TxB_2$ (□), Mmc-$TxB_2$ (■) and 4-Lum-$TxB_2$ (△)
(To be intelligible, only the dose response for $TxB_2$ was fitted).
(A) with $^{125}I$ $TxB_2$ RIA kit (B) with acetylcholinesterase $TxB_2$ EIA kit

recorded on a Model 5020 integrator from Spectra Physics.

*Spectral determinations*

Absorbance was measured with a Shimadzu UV-visible spectrophotometer (UV 160, Kyoto, Japan). $TxB_2$ esters were solubilized in 0.06*M* phosphate buffer (pH 7.3) making sure that the absorbance was close to 0.01.[17] Fluorescence quantum yields ($Q_f$) were calculated compared to a 1 mg/ml quinine hydrogenosulphate ($Q_f = 0.546$) solution prepared in 0.05*M* sulphuric acid.[17,18] The excitation and emission spectra, the quantum yields and the excited state life-time ($\tau$) were measured with a multi-frequency phase fluorometer (SLM-AMINCO 48000 S, SLM Instrument Inc., Il., U.S.A.). Fluorescence anisotropy was measured by a set-up developed in INSERM U 284 (Fluopolarimeter). The fluorescence anisotropy $\langle r \rangle$ is defined by the equation

$$\langle r \rangle = \frac{I\parallel - I\perp}{I\parallel + 2I\perp}$$

where $I\parallel$ and $I\perp$ represent the emission intensities polarized parallel and perpendicular to the direction of polarization of the exciting light, respectively. A blank subtraction was performed for each sample. The measurement cell was kept at constant temperature with a circulation water bath at 25° for the determination of anisotropy of $TxB_2$ derivatives in 0.06*M* phosphate buffer pH 7.3 or at 0°, 5°, 10°, 15° and 20° for the determination of probe fluorescence

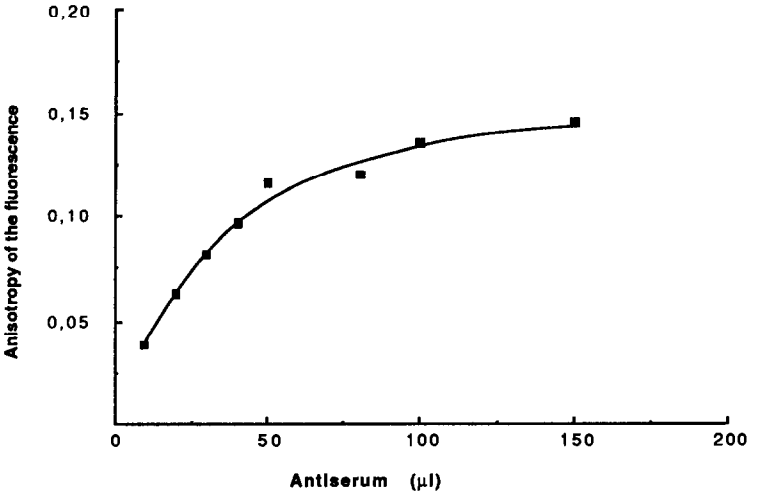


Fig. 4. Variation of fluorescence anisotropy (4-Lum-$TxB_2$) as a function of antiserum volume.

anisotropy in propyleneglycol. Viscosity of propyleneglycol was determined with a viscosimeter Low Shear 30 (Contraves, Zurich, Switzerland).

*Immunorecognition*

The principle of the immunoassays used is competitive binding. The standard curves were established according to the procedure guidelines of the manufacturers of the kits.

*$^3H$ RIA.* $TxB_2$, Mmc-$TxB_2$ and 4-Lum-$TxB_2$ (5, 10, 25, 50, 100 and 250 pg per tube) were assayed. Incubation with antiserum and tracer (total activity was 4400 cpm) was carried out overnight at 4°. Separation of bound from free labeled conjugate was made by adding a charcoal–dextran suspension followed by centrifugation at 1000 g for 10 min at 4°. An aliquot of 500 µl of the supernatant was mixed with 5 ml of scintillation liquid (Ready Safe cocktail, Beckman) and counted for 10 min (Beckman LS 6000-SC).

*$^{125}I$ RIA.* $TxB_2$, Mmc-$TxB_2$ and 4-Lum-$TxB_2$ (3.1, 6.2, 12.5, 25, 50, 100, 200 and 400 pg per tube) were assayed. Incubation with antiserum and tracer (total activity was 15,000 cpm) was carried out overnight at 4°. Then a homogenous suspension (500 µl) of Amerlex-M second antibody was added. After centrifugation at 1500 g for 10 min at 4°, the supernatant was discarded and the radioactivity was counted for 1 min (Beckman Gamma counter B 5 500).

*EIA.* $TxB_2$, Mmc-$TxB_2$ and 4-Lum-$TxB_2$ (7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1000 pg per well) were assayed. Incubation with antiserum and enzymic tracer (total activity: 2.240 A.U.) was performed overnight at 25°. Then, the wells were washed to remove any unbound reagents and Ellman reagent (which contains the substrate which reacts with acetylcholinesterase) was added. After incubation for 30 min at 25°, absorbance was read at 412 nm on a Titertek Multiskan MCC/340 (Flow Laboratories, Rickmansworth, U.K.).

Table 4. Variation of fluorescence anisotropy $\langle r \rangle$ in competition experiments ($n = 3$)

| $TxB_2$(ng) | $\langle r \rangle$ | RSD (%) |
|---|---|---|
| 0 | 0.121 | 6 |
| 0.5 | 0.120 | 10 |
| 1.0 | 0.110 | 6 |
| 2.0 | 0.072 | 8 |
| 3.0 | 0.042 | 5 |
| 5.0 | 0.040 | 8 |

*Competition experiments*

All reagents were diluted in 0.06*M* phosphate buffer pH 7.3. Each vial of antibody was reconstituted to a final volume of 0.5 ml. Solution of 4-Lum-$TxB_2$ (100 ng/ml) was prepared and stored at 4° for up to 2 weeks.

The dilution of antibody that allows a significant variation of anisotropy was determined first. Various volumes of antiserum were incubated with the same amount of 4-Lum-$TxB_2$ (5 ng) at 25° during 60 min in disposable acryle cuvettes (Sarsted, Molsheim, France). The volume was completed to 1.0 ml with the diluent buffer just before the measurement of fluorescence anisotropy.

Competition experiments were realized as follows: 4-Lum-$TxB_2$ derivative (50 µl) and

dilutions of standard $TxB_2$ (0.5, 1.0, 2.0, 3.0 and 5.0 ng) were incubated with antiserum (50 $\mu$l) for 60 min at 25°. The volume was completed to 1.0 ml with the diluent buffer just before the measurement of fluorescence anisotropy.

## RESULTS AND DISCUSSION

### *Derivatization*

Structure of the derivatives obtained with the different probes is shown on Fig. 1. Optimum conditions of derivatization have been determined and are summarized in Table 1. To make sure that the optimum amount of reagent was used, the peak area was investigated as a function of the reagent/$TxB_2$ molar ratio. In the case of esterification, as BrMmc and PBr react with the carboxylic function of $TxB_2$ a three-fold excess of reagent was sufficient, when for 7-CMMC a six-fold excess was necessary because of reaction with the three alcoholic functions. Successful derivatization of small amounts of carboxylic acids with 4-Lum was recently reported.[19] Amidification of the carboxylic function of this compound requires a specific activation step. For this purpose, carbodiimides has been widely used alone or in combination with various reagents, particularly *N*-hydroxysuccinimide.[20] In our case, activation was carried out by DCC in presence of HOBT as previously reported.[21]

### *Purity and stability of the derivatives*

As the fluorescent $TxB_2$ derivatives have to be used as labeled antigens in the development of an immunoassay, it was necessary to obtain highly pure and stable derivatives. Liquid chromatography can easily achieve their separation from the excess of reagent used during the synthesis. Reversed phase chromatography of derivatized prostaglandins is generally performed on octadecyl modified silica.[1,4] However water content in the mobile phase was a major drawback to the isolation of the ester derivatives since we established that hydrolysis of the ester bond occurred during freeze-drying of the collected fractions. Therefore, we developed normal phase semi-preparative and analytical chromatographic systems. The excess of reagent was efficiently separated from the corresponding $TxB_2$ derivative (Table 2) even when two peaks were obtained in the case of derivatization of the carboxylic function owing to the equilibrium between the aldehydic and the cyclic hemiacetal forms of the molecule. The solvents—hexane and ethanol—were further easily removed by evaporation. The purity of the dried fraction was 91% for Mmc-$TxB_2$, 93% for P-$TxB_2$, 98% for MMC-$TxB_2$ and 95% for 4-Lum-$TxB_2$ when determined by analytical HPLC.

Stability was tested by HPLC and measurement of the fluorescence anisotropy. $TxB_2$ derivatives were stored as dry residues at −20° and −70° or dissolved in 0.06*M* phosphate buffer (pH 7.3) at 4°. Any decrease in the labeled $TxB_2$ peak area measured by HPLC or in the fluorescence anisotropy can be related to the release of the fluorescent label. It has been noted that the stability is favoured by storage as dry residues at −70°. The derivatives are stable for up to six weeks at −70° and two weeks at −20°. In solution at 4°, stability of the esters is only one week, but three weeks for the amide, which corroborates the resistance to hydrolysis of the amide bound. After labeling, antigen must be water-soluble in order to be used in a homogenous assay. The labeling by the different probes preserves the hydrosolubility and the fluorescence intensity is not influenced by pH when tested in 0.06*M* phosphate buffer at pH 2.5, 4.5, 7.0, 7.3 and 7.8.

### *Spectroscopic properties of the derivatives*

The main characteristics of a fluorophore used as a label in fluoroimmunoassays are a high molar absorptivity, a high quantum yield, a large Stokes' shift (to eliminate scattering) with an emission maximum at a high wavelength (to minimize endogenous fluorescence). In the particular case of fluorescence polarization, the life-time of the excited state of the fluorophore must be considered. When a fluorescence molecule is excited with polarized light, the polarization of the emitted light depends on the extent of random Brownian rotation of the molecules. The life-time of the probe linked to antigen must be long enough to observe any rotation. For fluorescence anisotropy, the depolarization of the emitted light is estimated by the anisotropy value $\langle r \rangle$. In the case of a labeled antigen with a low molecular weight, rotation is fast and the anisotropy value is low. After binding to antibody, this value is increased.[22] It would then be possible to use polarization fluorescence in competition homogenous immunoassays for small antigens like $TxB_2$.

Table 3 summarizes the properties for each derivative of $TxB_2$. P-$TxB_2$ seems unsatisfactory considering a relatively low value of $Q_f$ (0.20)

and an anisotropy value much too high (0.240) leading to an impossible increase when the labelled antigen will bind to the corresponding antibody. In contrast, characteristics of Mmc-$TxB_2$, MMC-$TxB_2$ and 4-Lum-$TxB_2$ are promising. The fluorescence anisotropy measured at 25° in phosphate buffer is low (0.039–0.049), so we can expect an increase of this value when binding to the antibody. Simulation of the binding was estimated by measurement of the fluorescence anisotropy in a viscous medium like propyleneglycol. As expected, the values of anisotropy depend on the viscosity of propyleneglycol (Fig. 2).

4-Lum presents some specific advantages over the other probes:

—fluorescence is measured after excitation at a high wavelength (405 nm), which favours a minimization of interferences due to excitation of endogenous substances of the samples which generally occurred in the UV region
—the high quantum yield (0.46) and the high molar absorptivity (25000) must allow a low limit of detection
—a single value of life-time (9.3 nsec), in comparison with MMC-$TxB_2$ and P-$TxB_2$.

*Immunorecognition of $TxB_2$ derivatives*

Since radiolabeling with tritium does not alter the chemical structure of the antigen, the $^3H$ $TxB_2$ kit was initially used to check the recognition of the $TxB_2$ derivatives. Immunorecognition was estimated by the ratio of concentrations (standard $TxB_2$/derivatized $TxB_2$) which displaced 50% of the bound $^3H$ $TxB_2$. A ratio of 1 means identical immunoreactivity for derivatized and standard $TxB_2$. For 4-Lum, Mmc-$TxB_2$, P-$TxB_2$ and MMC-$TxB_2$ ratio values were 1.00, 0.90, 0.25 and 0.05 respectively. Lowest value is obtained with MMC-$TxB_2$ derivative after esterification of the three hydroxyl groups. It is likely that the employed antibody was raised against an immunogen couple at the carboxyl of $TxB_2$ and thus recognizes only $TxB_2$ derivatives with free hydroxyl groups.

4-Lum-$TxB_2$ and Mmc-$TxB_2$ present the best immunorecognition. Confirmation was obtained by use of $^{125}I$ $TxB_2$ and EIA kits. As labeling with iodine or acetylcholinesterase induces structural modification of $TxB_2$, we expect the steric hindrance to be of the same order of magnitude for the fluorescent- and the radio- or enzymic labeled $TxB_2$.[23] The results are shown in Fig. 3. Only the dose response curve for $TxB_2$ was fitted as reference. In comparison, the experimental values obtained with 4-Lum-$TxB_2$ and Mmc-$TxB_2$ are very similar and demonstrate that the immunorecognition of these tracers by the antiserum is well preserved.

*Competition experiments*

As 4-Lum-$TxB_2$ derivative exhibits good chemical stability and promising spectral and immunologic properties, we decided to explore the variations in fluorescence anisotropy under competition conditions.

Although the antisera were commercialized for radioimmunoassay, it was possible to use them and to study the modification of fluorescence anisotropy following the addition of increasing amounts of antiserum to a constant amount of 4-Lum-$TxB_2$. In the absence of antiserum, the tracer exhibits a low anisotropy value, $\langle r \rangle = 0.039$, typical of a rapid tumbling motion of a small molecular weight tracer in fluid medium. As antiserum is added, the fluorescence anisotropy increases with the antibody concentration (Fig. 4), demonstrating that the derivative is bound. A volume of 50 $\mu$l of antiserum was chosen for the competition experiments between various amounts of $TxB_2$ and a fixed amount of tracer (4-Lum-$TxB_2$) since it corresponds to a capacity binding near 50%. In this system, variation of anisotropy fluorescence is correlated to $TxB_2$ concentration (Table 4).

In conclusion, 4-Luminarine seems a valuable probe for labeling of $TxB_2$. However, the range of quantitation of $TxB_2$ by FPIA would be much higher than that observed with other immunoassays. This lack of sensitivity would be crucial in biological matrices since it is well known that the presence of proteins increases the diffusion of light and the noise level in homogenous immunoassays. Therefore, the field of determination of $TxB_2$ by FPIA will be *in vitro* pharmacological studies (concentration range 0.1–100 ng/ml) and previous extraction of $TxB_2$ will be recommended as usually performed in other works.[24]

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