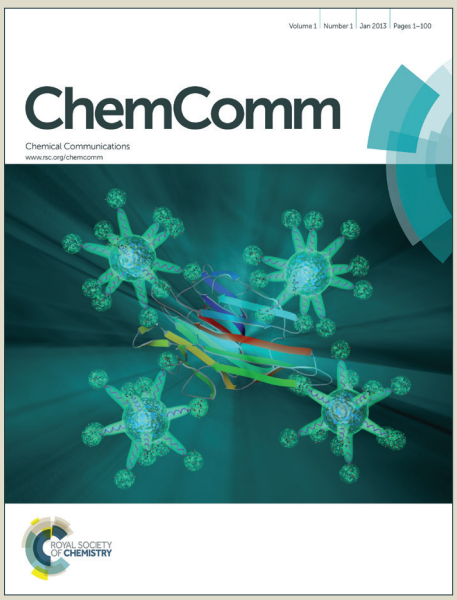
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

ChemComm

RSCPublishing

COMMUNICATION

Spiroguanidine rhodamines as fluorogenic probes for lysophosphatidic acid

Cite this: DOI: 10.1039/x0xx00000x

Lei Wang,[a] Martha Sibrian-Vazquez,[a] Jorge O. Escobedo,[a] Jialu Wang,[a] Richard G. Moore[b] and Robert M. Strongin[a,*]

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

Direct determination of total lysophosphatidic acid (LPA) was accomplished using newly developed spiroguanidines derived from rhodamine B as universal fluorogenic probes. Optimum conditions for the quantitative analysis of total LPA were investigated. The linear range for the determination of total LPA is up to 5 μM with a limit of detection of 0.512 μM.

Lysophosphatidic acids (LPAs) are a family of phospholipids characterized by a single fatty acyl chain, a glycerol backbone and a phosphate head group. LPAs stimulate specific, multiple processes on the cells of the vessel wall and activation of blood platelets through G protein-coupled receptors.[1,2] These include cell proliferation and migration, stress fiber and focal adhesion formation, fibronectin matrix assembly, collagen gel contraction, platelet aggregation, endothelial dysfunction, expression of cell adhesion molecules, and biochemical resistance to chemotherapy and radiotherapy-induced apoptosis.[3,4] As LPAs are important signaling molecules, their tight regulation by LPA-generating and LPA-inactivating enzymes indicate their potential involvement in certain human diseases that include cardiovascular disease,[5] neuropathic pain,[6] multiple sclerosis,[7] lung disease,[8] bone metastases,[9] diabetic retinopathy[10] and liver fibrosis.[11] Moreover, high levels of LPAs have been detected in ascitic fluid from ovarian cancer patients.[12] Clinical studies have suggested that specific LPA subspecies (Fig. 1) are associated with ovarian cancer.[13]

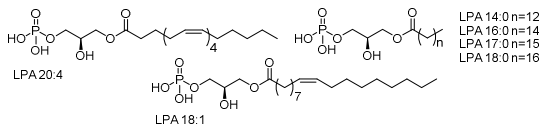


Fig. 1 Lysophosphatidic acids.

Common detection methods of phospholipids include gas chromatography (GC),[14] capillary electrophoresis (CE)[15] and two-dimensional thin layer chromatography (TLC).[16] High resolution liquid chromatography-mass spectrometry (LC-MS) has been developed for quantitative analysis of phospholipids.[17] However, it is not as accurate as liquid chromatography-tandem mass spectrometry (LC-MS/MS). Modern soft ionization techniques, such as electrospray and matrix-assisted laser desorption, have greatly improved phospholipid analysis in a faster and more accurate manner.[18] However, LC-MS/MS is not ideal for LPA analysis.[19,20] It is well documented that sample preparation for LC-MS/MS analysis results in some endogenous matrix, containing highly ionic components such as phosphatidyl choline (PC) and lysophosphatidyl choline (LPC), which may either suppress or enhance ionization in the electrospray MS source.[21] Furthermore, an important issue with using LC-MS/MS in the quantification of LPA is the cleavage of the choline head group from LPC which releases LPA and thereby produces false positives.[22] The matrix effects may also result in retention time shifts and elevated baselines that can affect the accuracy and reproducibility of quantification.[16,23] Optical detection, on the other hand, is a relatively simple and accessible method that can provide the required sensitivity for the detection and quantification of LPAs. Commercially available fluorogenic probes for phospholipids detection are fluorescent lipid analogs. Their use for phospholipids detection is based on the enhancement or quenching of fluorescence emission intensity as a result of aggregation/de-aggregation. DiA,[24] DPH,[25] DSHP,[26] nonyl acridine orange,[27] 3-hydroxyflavone derivatives,[28] and benzanthrone derivatives[29] have been widely used to investigate phospholipid behavior in both artificial and biological systems. Since aggregation/de-aggregation is dependent on the type, length and number of alkyl chains present in a specific phospholipid, the magnitude of a signal

for total phospholipids in a sample is influenced by specific component structures in addition to total phospholipid levels. Therefore, to obtain a more accurate signal corresponding to total LPA, fluorophore signal transduction should be independent of alkyl chain features.

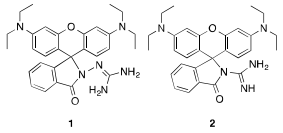
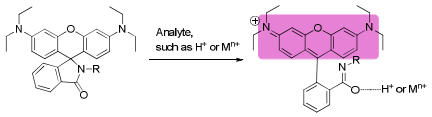


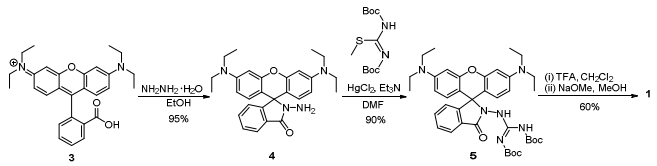
Fig. 2 Spiroguanidine rhodamines **1** and **2**.

Probes **1** and **2** (Fig. 2) are based on the rhodamine B framework functionalized with a guanidine group. They are non-fluorescent and colorless in the lactam form, whereas the corresponding open amide form is strongly fluorescent (Scheme 1).[30] The guanidinium moiety is a natural anion-binding receptor. Its unique combination of cationic charge and hydrogen-bond donor properties has inspired its use herein in LPA sensor design. Another attractive feature is the extremely high basicity of guanidine (pK_a = 13.5), which guarantees protonation over a wide pH range.[31] LPAs have two ionizable protons (pK_{a1} = 2.9 ± 0.3 and pK_{a2} = 7.47 ± 0.03).[32] The signal enhancement would thus be controlled mainly by the phosphate head group rather than the type, length and number of alkyl chains present in the phospholipid.



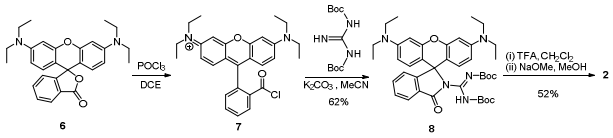
Scheme 1. Spirolactam ring opening of a rhodamine B derivative.

Compound **1** is synthesized in four steps starting from rhodamine B (Scheme 2). Rhodamine B hydrazide **4** is synthesized according to the procedure reported by Yang *et al.*[33] in 95% yield. Attempts to synthesize compound **1** via guanidinylation of **4** with cyanamide under acidic conditions were unsuccessful.[34] Alternatively, the use of the guanidinylation reagent, 1,3-*bis*-boc-2-methyl-2-thiopseudourea, and $HgCl_2$ as the catalyst following a modified procedure reported by Dardonville *et al.*[35] affords the di-*N*-Boc-protected guanidine **5** in 90% yield. After removal of the Boc protecting group and neutralization, compound **1** is obtained in 60% yield.



Scheme 2. Synthesis of spiroguanidine rhodamine **1**.

Rhodamine B acyl chloride **7** is obtained according to the procedure reported by Liu *et al.*[36] (Scheme 3). Initial attempts to obtain **2** via direct acylation reaction of guanidinium chloride with rhodamine B acyl chloride were unsuccessful. Guanidinylation of the rhodamine B lactam with 1,3-*bis*-boc-2-methyl-2-thiopseudourea to afford di-*N*-boc protected spiroguanidine rhodamine, was also unsuccessful.[37] As an alternative, the direct acylation reaction of 1,3-*bis*(tert-butoxycarbonyl)guanidine with **7** under basic condition was attempted. Following a modified procedure reported by of Liu *et al.*,[38] the di-*N*-boc-protected intermediate **8** is obtained in 62% yield. Upon Boc deprotection and neutralization, **2** is obtained in 52% yield.



Scheme 3. Synthesis of spiroguanidine rhodamine **2**.

Stock solutions of **1** and **2** were prepared in $CHCl_3$. Aliquots were transferred into 4 mL vials, and evaporated under a stream of N_2. Different solvent systems, including aqueous buffers at different pH values as well as DMSO, DMF, THF, $CHCl_3$, MeOH and EtOH, were evaluated by reconstitution of the dry probe with solvent. The absorption and fluorescence spectra of 5 μM solutions of **1** and **2** in $CHCl_3$:DMSO (9:1) show that these newly developed probes have similar maximum absorbance and emission wavelength as compared to the parent rhodamine B (Figs. S1 and S2, ESI†). The relative fluorescence quantum yields (φ) of **1** and **2** were calculated by using rhodamine B base as the reference standard, following the procedure of Würth *et al.*[39] Spectroscopic properties of **1** and **2** in EtOH are summarized in Table S1 (ESI†). High fluorescence emission intensity is observed for **2** in protic solvent systems, which is attributed to the open form (Scheme S1, ESI†), while **1** remains as the spirocyclic form with low fluorescence intensity. Probes **1** and **2** were evaluated for the detection and quantification of LPA. LPA 16:0 was chosen as a model analyte to carry out LPA fluorescence sensing experiments. Of the various solvent systems evaluated, the greatest fluorescence enhancement was observed in $CHCl_3$ upon treatment of both **1** and **2** with LPA. However, it was found that LPA 18:0, in its protonated form, exhibited only partial solubility in $CHCl_3$. Addition of DMSO improved solubility. In order to obtain more uniform samples for measurement, films of each LPA subspecies were prepared from their respective MeOH solutions. MeOH was evaporated under a stream of N_2, and the films reconstituted in $CHCl_3$:DMSO (9:1) for spectral analysis. Initial screening (Fig. 3) showed that **2** had a higher fluorescence emission in the presence of LPA 16:0 as compared to that of **1.**

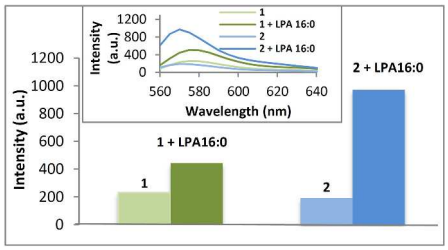


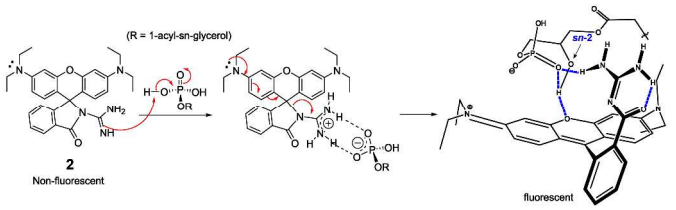
Fig. 3 Fluorescence emission intensity comparison between **1** and **2** in the presence of LPA 16:0. Ex/Em = 550 nm/570 nm; final probe concentration: 5 μM, final LPA 16:0 concentration: 5 μM; solvent system: $CHCl_3$: DMSO 9:1.

In order to prove that the fluorescence enhancement is independent of the degree of unsaturation and length of alkyl chains present in the phospholipids, fluorescence measurements of **2** with different LPA subspecies were carried out. As shown in Fig. S4 (ESI†), equimolar solutions (10 μM) of different LPA subspecies resulted in the same fluorescence emission intensity upon interaction with **2** (10 μM). Upon treating an LPA solution with **2**, enhanced fluorescence intensity was instantly observed and as shown in Fig. S5 (ESI†). The fluorescence intensity of each subspecies remained constant for at least 30 min. Based on the preliminary positive results described above, the performance of **2** for LPA detection was optimized. From the screening of a series of solvent systems (*e.g.*, Fig. S5, ESI†), it was found that a $CHCl_3$:DMSO 95:5 mixture produced the best results in terms of fluorescence enhancement. To avoid potential ring opening and interference due to the presence of acids, the solvents were passed through anhydrous K_2CO_3.

A Job's plot (Fig. S6, ESI†) was conducted keeping [**2**] + [LPA] = 10 μM. The inflection point was found at 0.5, indicating a 1:1 stoichiometry for **2** and LPA (ESI†). The binding constant (*Ka*) for the interaction of **2** and LPA was determined to be 4.622×10^5 M^{-1}. The concentration of LPA in the protonated form (free acid), was determined by LC-ESI/MS/MS using LPA 17:0 as an internal standard. Calibration curves of individual LPAs were obtained over a 1-15 μM range (Fig. S7, ESI†). Acceptable correlation factors (R^2) were obtained for all the LPAs (Table S2, ESI†). Solutions of increasing concentrations (0, 0.5, 1, 3, and 5 μM) of LPA subspecies were prepared in a mixture of MeOH:$CHCl_3$ 1:1. After evaporation under N_2, samples were reconstituted with a $CHCl_3$:DMSO (95:5) mixture. Emission intensity for each solution was recorded from 560 to 700 nm using an excitation wavelength of 550 nm. As shown in Fig. S9 (ESI†), a linear relationship between fluorescence and LPA concentration in the range of 0-5 μM was obtained with a limit of detection (LOD) of 0.512 μM. This value is considered suitable for the quantification of total plasma LPA levels from both healthy and ovarian cancer patients. According to a clinical study,[40] the mean preoperative total plasma LPA levels are below 1 μM in healthy women, and between 1 and 5 μM in ovarian cancer patients.

The proposed LPA sensing mechanism involving **2** is based on the conversion from a spirocyclic guanidinium system to a ring-opened form assisted by the interaction of the phosphate group on LPA. Extensive studies of the guanidinium group as a receptor for phosphate are well documented in the literature. It has been found that phosphate can bind through an extensive hydrogen-bonding network as well as through the formation of a salt bridge with the guanidinium moiety.[41] This type of structural complex has been found in many crystal structures of enzyme complexes with phosphate substrates as well as in simple guanidinium salts.[42]

The mechanism is envisioned to begin via an intermolecular proton transfer from the LPA phosphate to the guanidine moiety resulting in the formation of a guanidinium salt (Scheme 4).



Scheme 4. Proposed LPA sensing mechanism with spiroguanidine rhodamine **2**. Non-fluorescent **2** gives rise to fluorescence emission upon LPA binding.

The carbonyl adjacent to the protonated guanidine group weakens the spirolactam, thus promoting conversion of **2** to a more delocalized xanthene dye via ring opening. The resultant positively charged fluorescent xanthene form is further stabilized via interaction with the polar anionic head group of LPA. Molecular simulations (Sybyl-X™ version 2.0, Certara) show that in addition to the electrostatic interaction between the phosphate group and the xanthene diethyliminium group, there are two hydrogen-bonding interactions that reinforce the non-covalent binding of LPA with the open form of **2**. One is formed between the central bridging oxygen of the fluorophore and the *sn*-2 hydroxyl group of LPA and the second between the phosphate and guanidine groups (scheme 4).

Conclusions

Two universal fluorogenic probes (**1** and **2**) have been developed for LPA analysis based on spiroguanidine derivatives of rhodamine B. Probe **2** was found to afford a relatively brighter signal in the presence of LPA. The consistent response of **2** to each of the most abundant LPAs, despite their structural variability, simplifies LPA determination by precluding the need to calibrate each individual LPA subspecies. Because the magnitude of the fluorescence signal corresponds to total phospholipid levels and is not dependent on specific component structures (e.g. type and length of the fatty acyl chain), this method may be applied to the quantification of total LPA levels in conjunction with selective LPA isolation from bodily fluids. According to a procedure recently reported by Wang *et al.*,[24] relatively more abundant phospholipids can be readily removed affording a mixture containing the most significant five bioactive LPA subspecies as the major components. Ongoing work in our group

includes the use of **2** and analogs for (i) key components of sensors for direct measurement of LPA levels without mass spectrometry and HPLC separations, and (ii) to create chemosensors with enhanced selectivity for individual LPA subspecies.

Acknowledgments

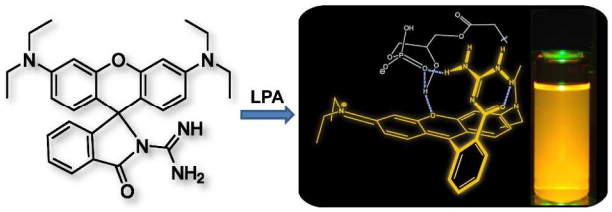
This work was supported by the National Institutes of Health (grant R01CA136491).

Notes and references

[a]*Department of Chemistry, Portland State University, Portland, OR 97201, USA. E-mail: strongin@pdx.edu; Tel: +1-503-725-9724*
[b]*Women and Infants Hospital, Brown University, 101 Dudley Street, Providence, RI 02905, USA. E-mail: RMoore@wihri.org; Tel: +1-401-453-7520*

†Electronic Supplementary Information (ESI) available: Detailed experimental procedures and chararcterization data of new compounds. See DOI: 10.1039/b000000x/

1 G. Tigyi, *Prostaglandins Other Lipid Mediat.*, 2001, **61**, 47.
2 J. W. Choi, D. R. Herr, K. Noguchi, Y. C. Yung, C.-W. Lee, T. Mutoh, M.-E. Lin, S. T. Teo, K. E. Park, A. N. Mosley and J. Chun, *Annu. Rev. Pharmacol. Toxicol.*, 2010, **50**, 157.
3 E. J. Goetzl, H. Dolezalova, Y. Kong, Y.-L. Hu, R. B. Jaffe, K. R. Kalli and C. A. Conover, *Cancer Res.*, 1999, **59**, 5370.
4 M. Bektas, S. G. Payne, H. Liu, S. Goparaju, S. Milstien and S. Spiegel, *J. Cell Biol.*, 2005, **169**, 801.
5 S. S. Smyth, H. Y. Cheng, S. Miriyala, M. Panchatcharam and A. J. Morris, *Biochim. Biophys. Acta*, 2008, **1781**, 563.
6 H. Ueda, H. Matsunaga, O. I. Olaposi and J. Nagai, *Biochim. Biophys. Acta*, 2013, **1831**, 61.
7 J. W. Choi and J. Chun, *Biochim. Biophys. Acta*, 2013, **1831**, 20.
8 Y. Zhao and V. Natarajan, *Cell. Signal.*, 2009, **21**, 367.
9 O. Peyruchaud, R. Leblanc and M. David, *Biochim. Biophys. Acta*, 2013, **1831**, 99.
10 A. Abu El-Asrar, G. Mohammad, M. Nawaz, M. Siddiquei, D. Kangave and G. Opdenakker, *Acta Diabetol.*, 2013, **50**, 363.
11 N. Watanabe, H. Ikeda, K. Nakamura, R. Ohkawa, Y. Kume, J. Aoki, K. Hama, S. Okudaira, M. Tanaka, T. Tomiya, M. Yanase, K. Tejima, T. Nishikawa, M. Arai, H. Arai, M. Omata, K. Fujiwara and Y. Yatomi, *J. Clin. Gastroenterol.*, 2007, **41**, 616.
12 Y. Xu, Z. Z. Shen, D. W. Wiper, M. Z. Wu, R. E. Morton, P. Elson, A. W. Kennedy, J. Belinson, M. Markman and G. Casey, *JAMA, J. Am. Med. Assoc.*, 1998, **280**, 719.
13 I. Sedlakova, J. Vavrova, J. Tosner and L. Hanousek, *Ceska Gynekol.*, 2006, **71**, 312.
14 J. Aranda, R. Motiejunaite, E. Im and A. Kazlauskas, *Diabetes*, 2012, **61**, 1225.
15 Y. L. Chen and Y. Xu, *J. Chromatogr. B Biomed. Sci. Appl.*, 2001, **753**, 355.
16 Y. Xiao, B. Schwartz, M. Washington, A. Kennedy, K. Webster, J. Belinson and Y. Xu, *Anal. Biochem.*, 2001, **290**, 302.
17 C. Wang, H. Kong, Y. Guan, J. Yang, J. Gu, S. Yang and G. Xu, *Anal. Chem.*, 2005, **77**, 4108.
18 M. R. M. Domingues, A. Reis and P. Domingues, *Chem. Phys. Lipids.*, 2008, **156**, 1.
19 G. Tigyi and A. L. Parrill, *Prog. Lipid Res.*, 2003, **42**, 498.
20 I. Fu, E. J. Woolf and B. K. Matuszewski, *J. Pharm. Biomed. Anal.*, 1998, **18**, 347.
21 Z. Zhao and Y. Xu, *J. Chromatogr. B*, 2009, **877**, 3739.
22 J. M. Onorato, P. Shipkova, A. Minnich, A.-F. Aubry, J. Easter and A. Tymiak, *J. Lipid Res.*, 2014, **55**, 1784.
23 H.-Y. Kim, T.-C. L. Wang and Y.-C. Ma, *Anal. Chem.*, 1994, **66**, 3977.
24 J. Wang, M. Sibrian-Vazquez, J. O. Escobedo, M. Lowry, L. Wang, Y. H. Chu, R. G. Moore and R. M. Strongin, *Analyst*, 2013, **138**, 6852.
25 I. Hurjui, A. Neamtu and D. O. Dorohoi, *J. Mol. Struct.*, 2013, **1044**, 134.
26 W. Zhao, W. Liu, W. Zhang, L. Zeng, Z. Fan, J. Wu and P. Wang, *Analyst*, 2012, **137**, 1853.
27 M. E. Rodriguez, K. Azizuddin, P. Zhang, S.-m. Chiu, M. Lam, M. E. Kenney, C. Burda and N. L. Oleinick, *Mitochondrion*, 2008, **8**, 237.
28 T. Ozturk, A. S. Klymchenko, A. Capan, S. Oncul, S. Cikrikci, S. Taskiran, B. Tasan, F. B. Kaynak, S. Ozbey and A. P. Demchenko, *Tetrahedron*, 2007, **63**, 10290.
29 O. Zhytniakivska, V. Trusova, G. Gorbenko, E. Kirilova, I. Kalnina, G. Kirilov and P. Kinnunen, *J. Lumin.*, 2014, **146**, 307.
30 H. N. Kim, M. H. Lee, H. J. Kim, J. S. Kim and J. Yoon, *Chem. Soc. Rev.*, 2008, **37**, 1465.
31 C. Hannon and E. Anslyn, in *Bioorganic Chemistry Frontiers*, eds. H. Dugas and F. Schmidtchen, Springer Berlin Heidelberg, 1993, vol. 3, pp. 193.
32 E. E. Kooijman, K. M. Carter, E. G. van Laar, V. Chupin, K. N. J. Burger and B. de Kruijff, *Biochemistry*, 2005, **44**, 17007.
33 H. Zheng, X.-Q. Zhan, Q.-N. Bian and X.-J. Zhang, *Chem. Commun.*, 2013, **49**, 429.
34 M. Sibrian-Vazquez, I. V. Nesterova, T. J. Jensen and M. G. H. Vicente, *Bioconjug. Chem.*, 2008, **19**, 705.
35 C. Dardonville, P. Goya, I. Rozas, A. Alsasua, M. I. Martin and M. J. Borrego, *Bioorg. Med. Chem.*, 2000, **8**, 1567.
36 W. Liu, L. Xu, H. Zhang, J. You, X. Zhang, R. Sheng, H. Li, S. Wu and P. Wang, *Org. Biomol. Chem.*, 2009, **7**, 660.
37 Z.-Q. Hu, C.-s. Lin, X.-M. Wang, L. Ding, C.-L. Cui, S.-F. Liu and H. Y. Lu, *Chem. Commun.*, 2010, **46**, 3765.
38 W. Liu, J. Chen, L. Xu, J. Wu, H. Xu, H. Zhang and P. Wang, *Spectrochim. Acta. A. Mol. Biomol. Spectrosc.*, 2012, **85**, 38.
39 C. Würth, M. Grabolle, J. Pauli, M. Spieles and U. Resch-Genger, *Anal. Chem.*, 2011, **83**, 3431.
40 T. Bese, M. Barbaros, E. Baykara, O. Guralp, S. Cengiz, F. Demirkiran, C. Sanioglu and M. Arvas, *J. Gynecol. Oncol.*, 2010, **21**, 248.
41 H. Luecke and F. A. Quiocho, *Nature*, 1990, **347**, 402.
42 G. Müller, J. Riede and F. P. Schmidtchen, *Angew. Chem., Int. Ed.*, 1988, **27**, 1516.



Fluorescent indicators have been developed that enable the determination of lysophosphatidic acids via signaling that is independent of the size or degree of unsaturation of the fatty acid side chain.