Organic & Biomolecular Chemistry

PERSPECTIVE

Alkoxyamines: a new family of pro-drugs against cancer. Concept for theranostics

Cite this: DOI: 10.1039/c3ob42076k

Gérard Audran,[a] Paul Brémond,[a] Jean-Michel Franconi,[b] Sylvain R. A. Marque,*[a] Philippe Massot,[b] Philippe Mellet,[b,c] Elodie Parzy[b] and Eric Thiaudière[b]

Received 17th October 2013,
Accepted 21st November 2013

DOI: 10.1039/c3ob42076k

www.rsc.org/obc

Development of anti-cancerous theranostic agents is a vivid field. This article describes a theranostic approach that relies on the triggering of cancer cell death by generation of alkyl radicals *at the right place and at the right time* using the presence of active proteases in the tumour environment. Alkoxyamines ($R^1R^2NOR^3$) are labile molecules that homolyze into nitroxides ($R^1R^2NO^{\bullet}$) and reactive alkyl radicals ($R^{3\bullet}$). They are used as a source of active alkyl radicals for curing and nitroxides for monitoring by Overhauser-enhanced magnetic resonance imaging (OMRI). Herein, the requirements needed for applying alkoxyamines are described: (i) highly selective activation of the alkoxyamine by specific proteases; (ii) fast homolysis of the alkoxyamine C–ON bond at physiological temperature; (iii) activation of cell death processes through local oxidative stress or potential re-activation of the immune system due to short-lived alkyl radicals; and (iv) imaging of the tumor and the drug release by sensing the nitroxide by OMRI.

Introduction

For 50 years now, cancer has been a major issue of public health in developed countries. Its occurrence has been steadily increasing due to lifestyle, environmental conditions and ageing. This has prompted tremendous efforts in many fields: fundamental biology, medical research, drug development, and prevention. Despite these efforts, huge funding and some success in curing, cancer remains a mostly unresolved issue regarding both its occurrence and its lethality, as shown by the number of recent statistics.[1] Over the last 50 years, cancer curing cases have increased due to clinical research and several therapeutic options. The current trend is to develop more specific drugs exhibiting much higher selectivity in discriminating between anomalous and healthy cells *in vivo*.[2]

Interestingly, recent research has indicated that cancer cells exhibit much higher, but still controlled, reactive oxygenated species (ROS, which are oxygen centered radicals or their precursors) activity than healthy cells. This has given rise to the idea that increasing the ROS/AO (anti-oxidants) ratio may lead to the death of cancer cells, either by apoptosis or by necrosis,[3] and their removal by phagocytic immune cells. In addition, excess radicals in cancer cells should lead to membrane protein and lipid modifications that trigger immune responses and ultimately cancer cell death.[4] Thus, increasing the amount of radicals in cells may therefore be applied as a therapeutic approach as long as they are generated *at the right place and at the right time* (Fig. 1, left part). However, the success of this approach relies on very drastic requirements that include carefully controlled generation of radicals in the cancer cell environment (controlled kinetics of their generation), and low cytotoxicity of the radical pro-drugs.[5] The main advantages of such an approach are that it provides the recruitment of immune cells and that it is applicable to a broad variety of solid tumors.[6] The needed selectivity may be achieved by activating the pro-drug *in situ* by X-/γ-ray irradiation, high intensity focused ultra-sound or by re-routing the redox status. However, it will be preferentially achieved through a specific enzymatic activity, for instance by taking advantage of the persistent matrix metalloprotease (MMP) activity linked to solid tumours, implying that the activation of the pro-drug will be strictly conditioned to this MMP activity. Indeed, the role of MMPs in tumour growth and metastasis has been studied for decades and is now clearly established.[7] MMPs overexpression originates not only from tumour cells but also from tumour-

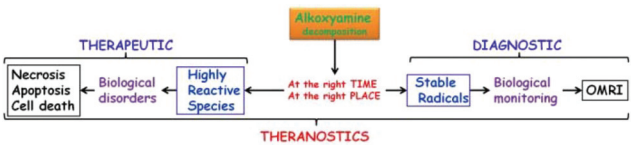

Fig. 1 General concept using alkoxyamines as theranostic agents.

[a]*Université d'Aix-Marseille CNRS – UMR 7273, case 551, Avenue Escadrille Normandie-Niemen, 13397 Marseille cedex 20, France. E-mail: sylvain.marque@univ-amu.fr*
[b]*CRMSB, CNRS-UMR-5536, Université Victor Segalen Bordeaux 2, 146 rue Léo Saignat, Case 93, 33076 Bordeaux cedex, France*
[c]*INSERM, 146 rue Léo Saignat, Case 93, 33076 Bordeaux cedex, France*

This journal is © The Royal Society of Chemistry 2014

associated stromal cells.[8] The success of anti-protease therapies with HIV proteinase inhibitors prompted research on MMP inhibitors to target tumour growth and metastasis.[9] Small inhibitors have been developed and some of them have reached the clinical trial step,[10] but all have failed.[11] The first obvious reason is insufficient basic knowledge of the biological role of each MMP involved in the tumour microenvironment. Most studies reveal a complex and versatile network of activated MMPs which is difficult to investigate *in vivo* (targeting the wrong MMP can have detrimental effects). For instance the activity of some MMPs seems to have a protective effect against tumour growth. The second difficulty is that *ca.* 30 MMPs, out of the 186 annotated in the human genome, are currently being studied.[12] This makes it difficult to synthesize specific inhibitors and accounts for the severe side-effects induced by the available drugs. Furthermore some MMP substrate specificities are provided by their non-catalytic domains[13] through their specific interactions with large substrates and cannot be reproduced with a low molecular weight inhibitor. Contrary to anti-protease therapies,[9] the concept described hereafter aims at using the specific activity of these MMPs as a specific trigger of alkoxyamine pro-drugs able to generate radicals *where and when such MMPs are present*.

During the last decade, a new concept known as *theranostics*[14] combining *thera*peutic (chemotherapy, gene therapy, *etc*.) and diag*nostic* (MRI, PET, *etc*.) abilities of suitable molecules has arisen. It aims to develop new molecules exhibiting therapeutic properties to cure diseases and spectroscopic properties to monitor the curing progress.[15] However, this approach mainly relies on the development of nanoparticles exhibiting both abilities. Nevertheless, nanoparticles suffer from several drawbacks such as difficulties in diffusing into tissues and in crossing the blood–brain barrier, a low clearance, causing the accumulation of nanoparticles in some organs and tissues leading to undesired cytotoxicity.[16] Hereafter, an alternative approach that relies on quickly eliminated small molecules and on a selectivity based on an enzymatic activity that releases highly reactive radical species is presented.

Magnetic resonance imaging (MRI) is a powerful non-invasive modality to investigate/monitor/detect diseases *in vivo*. Unfortunately, the sensitivity of MRI is intrinsically not sufficient for molecular imaging, which prompted the use of high polarization fields. The MRI signal can also be enhanced by dynamic nuclear polarization (DNP)[17] *in situ* through the Overhauser effect. It has recently been shown that 3D OMRI of tumour-bearing mice at a constant field of 0.2 T could be used to reveal the presence of stable nitroxides in the blood system and in a brain tumour (Fig. 2).[18] In this example, Overhauser enhancements were in the range of 2 to 5 at the tumour site, corresponding to nitroxide concentrations of 1 mM or lower. The same OMRI system was also used *in vitro* to demonstrate that a biochemical event such as proteolysis could be detected due to an Overhauser switch from a nitroxide-labelled protein before hydrolysis to OMRI detectable proteolysis products.[18]

Applied to neutrophile degranulation, this method proved capable of detecting faint elastolytic activity *ex vivo*.[18] *In vivo* detection of proteolysis based upon OMRI detection of nitroxides would rely on the generation of nitroxide faster than its elimination processes (bio-reduction, physiological clearance, *etc*.) to ensure a local nitroxide concentration high enough to afford an efficient MRI acquisition of this biological process. The actual device at 0.2 T provides a good contrast around 0.1 mM and devices at lower fields are expected to be more sensitive.

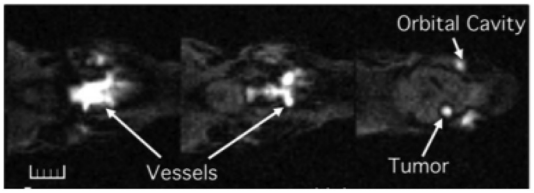

Fig. 2 Example of OMRI at 0.2 T. (Reprinted with permission from ref. 18. Copyright 2012 Wiley Interscience.)

For decades, free radicals have been considered as detrimental species for biological systems,[19] although they play key roles in the cell machinery.[20] Their involvement in living systems requires a tough control of their reactivity, which is brought by the presence of various anti-oxidants (AOs). However, randomly-generated harmful radicals[21] alter the membranes[20] – by abstraction of labile hydrogen atoms from proteins and lipids, by additions onto unsaturated lipids, *etc*. – and/or the nucleic acids; and this is known to trigger necrosis or apoptosis mechanisms (Fig. 1).[22] Consequently, approaches based on tougher control have been developed to limit the detrimental effects of an excess of radicals in cells.[23] However, in the last few years,[2,6] the use of radicals has been revived on the grounds of generation of radicals *at the right time and at the right place*. The latter would lead to beneficial applications of radicals for curing various diseases or pathologies.[3,6] For the last 3 decades, several types of molecules releasing highly active radicals have been proposed as drugs or pro-drugs for radiotherapy[24] or chemotherapy applications.[3]

However, none of them exhibit the same high potential as the alkoxyamines $R^1R^2NOR^3$ for controlling the generation of radicals or for selective modes of activation as well as contrasting/enhancing agents for MRI.

Alkoxyamines have been known since the late 20s[25] but their radical reactivity was only reported in 1974 by Kovtun *et al.*[26] (Fig. 4a), *i.e.*, homolysis of the C–ON bond to release an alkyl radical and a nitroxide. Their first valuable application has been proposed by Solomon *et al.*[27] with the wide use of these molecules both as initiators and as controllers for nitroxide mediated polymerization (NMP).[28]

The use of alkoxyamines as drugs or pro-drugs for therapy based on the radical reactivity requires handling molecules stable enough for long term storage whereas radical reactivity needs alkoxyamines undergoing fast C–ON homolysis under mild conditions (35–40 °C). These requirements led us to propose the concept of smart alkoxyamines (red dashed arrow), considering 3 families (Fig. 3b): one with bond dissociation energy (BDE) > 140 kJ mol^{-1} – unsuitable for radical

 This journal is © The Royal Society of Chemistry 2014

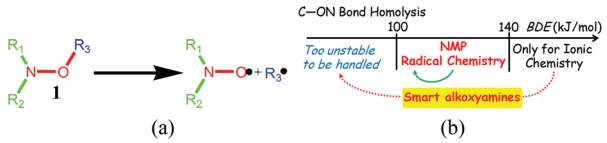

Fig. 3 (a) Homolysis of an alkoxyamine. (b) Outline of a smart alkoxyamine.

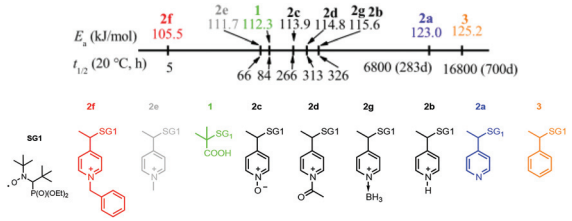

Fig. 4 Activation energies and half-life times $t_{1/2}$ at 20 °C for the C–ON bond homolysis of a series of chemically activated alkoxyamines.

chemistry, one with BDE between 100 kJ mol^{-1} and 140 kJ mol^{-1} – suitable for radical chemistry and NMP, and one with BDE < 100 kJ mol^{-1} – "*useless*" due to very low BDE, implying that the molecules are too unstable at room temperature for carefree handling except for the concept described in this *Perspective*.

Recently, we investigated various types of chemical activation (Fig. 4) of alkoxyamine **2a**, and we observed that the half-life time $t_{1/2}$ = 6800 h for **2a** decreased to $t_{1/2}$ = 66 h for **2e** in *t*-BuPh[29] at 20 °C (Fig. 4) and to $t_{1/2}$ = 48 min in water at 37 °C (physiological conditions).† This shows clearly that *alkoxyamines have potential applications as drugs and pro-drugs, and that the homolysis rate can be readily controlled and tuned to comply with the pharmacokinetic requirements* (green arrow in Fig. 3b).

Thus, using alkoxyamines for theranostics requires the development of alkoxyamines stable enough to be easily handled at room temperature (BDE > 140 kJ mol^{-1}) and capable of being activated into highly labile species (BDE < 100 kJ mol^{-1}, red dashed arrow in Fig. 3) which release alkyl radicals for therapeutic applications and nitroxides for diagnostic applications. Hereafter, we present the requirements and the evidence that alkoxyamines can be used as drugs or pro-drugs and also as agents for DNP-enhanced MRI (OMRI) for curing and monitoring various diseases, that is, as theranostic agents.

Alkoxyamines as theranostic agents: the concept

The chemical design is based on stable alkoxyamines made up of three distinct parts (Fig. 5): the trigger (yellow circle), the alkyl fragment (green square) and the nitroxide fragment (blue triangle). The trigger is sensitive to the targeted biological activity and releases a transient labile alkoxyamine (green square and blue triangle in Fig. 5). The latter undergoes fast homolysis into a highly reactive alkyl radical (yellow square) and a persistent nitroxide (blue triangle) at physiological temperature. The alkyl radical will play the role of a therapeutic agent by altering cell surface proteins and lipids and inducing oxidative stress, relying on the concept described in Fig. 1. The nitroxide will be involved as an MRI signal enhancer *via* the Overhauser effect (*vide supra*). Then, it will be possible to monitor the location of the disease-associated biochemical reaction and its evolution and to monitor the local pro-drug to drug activation and thus to measure the release of the drug *in situ*.

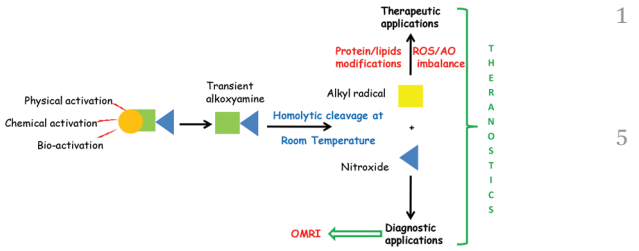

Fig. 5 Pre-requisites for the theranostic application of radicals as drugs and as monitoring species.

This very simple use of alkoxyamines still has to comply with several requirements which are general for the theranostic applications and some others which are specific to their therapeutic and diagnostic applications. The molecules developed for theranostic applications aim at being easily handled during clinical practice as well as being therapeutically efficient. Thus, the pro-drugs should be highly stable and capable of being eliminated rapidly by renal clearance if not converted *in situ* into an active drug. As the alkoxyamines are made up of 3 parts, several approaches are available to fulfil the pre-requisites.

Approaches for the activation

The homolysis of alkoxyamines can be triggered by various processes: physical, chemical, and biological (Fig. 6). The choice of the process will depend on the target, the aim and the therapy procedure.

Physical activation. (i) It can be performed by increasing the temperature which is the procedure currently applied in the field of polymer chemistry. However, for biological systems, this increase is limited to 40 °C for physiological reasons although higher temperatures are allowed for external skin applications; (ii) it has been shown that the homolysis of suitable alkoxyamines can be initiated by UV-visible irradiation.[30] This approach is only suitable for external (skin) or some internal applications. Some examples of alkoxyamines homolysis by ultra-sound are known.[31] Consequently, it should be possible to apply sonochemical initiation using focalized ultrasound for selective highly localized homolysis; (iii) the radiochemical (X-/γ-ray) irradiation is currently applied against

† E_a = 106.8 kJ mol^{-1}, A = 2.4 10^{14} s^{-1}.

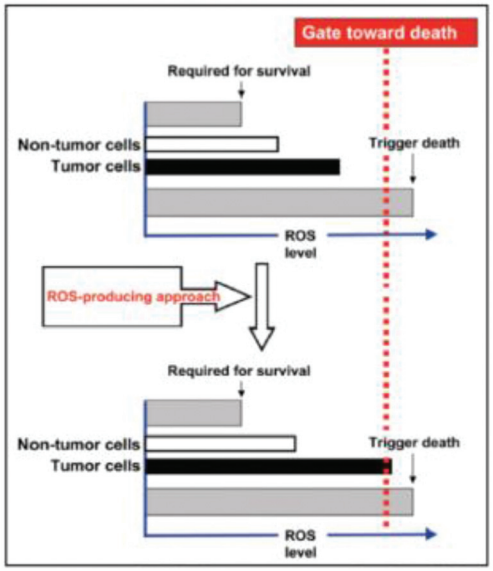

Fig. 6 Concept for the threshold of ROS/AO ratio that triggers cellular death processes. (Reprinted with permission from ref. 3. Copyright 2008 Landes Bioscience.)

certain cancers.[24] It relies on the cleavage of weak bonds leading to radicals which scavenge the oxygen solved in blood to generate ROS, which trigger the mechanisms of cellular death. One may expect that the X-/γ-ray irradiations of molecules with weaker bonds would require less intensive and/or shorter irradiation time while keeping the same curing efficiency.

Chemical activation. As displayed in Fig. 4, several modes of activation, *e.g.*, oxidation, methylation, *etc*., can be used to trigger the alkoxyamine homolysis.

In our opinion, a high selectivity will be preferentially achieved by combination with a biological triggering (*vide infra*). Nevertheless, a non *selective approach* (*vide infra*) of activated alkoxyamines by a chemical reaction can be applied (*e.g.* Fig. 4). It is likely that other modes of activation might be suitable.

Biological activation. As mentioned above, the partial digestion of the drug by the targeted proteases would release a transient alkoxyamine whose homolysis can be enhanced by protonation or chemical rearrangement.

Approaches for therapeutics

Depending on the mode of activation, two therapeutic approaches are possible: the "*selective*" and the "*non-selective*" approach. The choice will depend both on the targets, the aims, and the preparation requirements.

"Selective" approach. It requires the combination of efficient drug addressing and chemical activation. To increase the chance of success, it is possible to use proteases present in the tumour micro-environment, for example MMPs. In such a case, only when the drug reaches the tumour, it will be converted to a transient alkoxyamine activated, either by protonation of a suitable amine function or by rearrangement into a more reactive group, and will release the alkyl radicals triggering the cell death. Since the molecular weight of a typical peptide-alkoxyamine pro-drug will not exceed 2000 D, the excess of unreacted pro-drug will be eliminated by renal clearance.

"Non-selective" approach. In such a case, the drug addressing would not have been possible, the efficiency of the alkoxyamine as drugs would rely on the concept assuming that tumoral cells contains an amount of oxidant species higher than that in healthy cells but still lower than the threshold required to trigger cellular death.[3] Then, increasing the amount of radicals in the cell would increase the imbalance of the ROS/AO ratio in such a way that the level of ROS would be high enough to cross the lethal threshold in tumoral cells whereas it would be almost innocuous for healthy cells (Fig. 6). Thus, only unhealthy cells exhibiting a high level of ROS would be sensitive to the excess of generated radicals (Fig. 6). The other cells would stand the increase in ROS by generating only little stress. Such an approach offers a larger choice of structural designs of alkoxyamines as well as of the activation modes.

Requirements for diagnostic applications and drug release monitoring

To be observed by OMRI or EPRI, the released nitroxide must be generated at a high rate to afford a steady state concentration in the tumor environment suitable for detection (around 0.1 mM with our current device as seen above). This means both efficient activation by physical or biological means and fast homolysis ($t_{1/2}$ in the range of a few minutes).

Conclusion

At this time, preliminary results show that alkoxyamine homolysis is possible at physiological temperature and also that selective triggering of alkoxyamine homolysis is now possible. OMRI can be used to monitor the latter process and the generation of extra radical species in the cell can initiate the cell death. It is obvious that the translation of this concept in the clinical world is still a long way off. However, due to the core structure (alkoxyamine $R^1R^2NOR^3$) of the drug, which exhibits a high versatility as for R^1, R^2, and R^3 groups, this family of molecules exhibits a high potential for applications in biological systems and for the development of new approaches based on radical chemistry.

Work is in progress to design a suitable model to target glioma, a specific type of brain cancer. However, this concept is very broad, and has the potential to be applied to many types of tumours and diseases.

Acknowledgements

Authors are grateful to ANR (grants 11-JS07-002-01 and 09-BLAN-0017-01) as well as Aix-Marseille University, CNRS,

 This journal is © The Royal Society of Chemistry 2014

INSERM, and University of Bordeaux 2 Victor Segalen for financial support.

Notes and references

1 J. Ferlay, E. Steliarova-Foucher, J. Lortet-Tieulent, S. Rosso, J. W. W. Coebergh, H. Comber, D. Forman and F. Bray, *Eur. J. Cancer*, 2013, **49**, 1374–1403.

2 T. Ozben, *J. Pharm. Sci.*, 2007, **96**, 2181–2196.

3 J. Wang and J. Yi, *Cancer Biol. Ther.*, 2008, **7**, 1875–1884.

4 C. J. Binder, *Adv. Exp. Med. Biol.*, 2012, **750**, 2–13.

5 A. S. Karwa, A. R. Poreddy, B. Asmelash, T.-S. Lin, R. B. Dorshow and R. Rajagopalan, *ACS Med. Chem. Lett.*, 2011, **2**, 828–833.

6 D. Trachootham, J. Alexandre and P. Huang, *Nat. Rev. Drug Discovery*, 2009, **8**, 579–591.

7 R. L. Scherer, J. O. McIntyre and L. M. Matrisian, *Cancer Metastasis Rev.*, 2008, **27**, 679–690.

8 A. Noël, M. Jost and E. Maquoi, *Cell Dev. Biol.*, 2008, **19**, 52–60.

9 Proteinase as Drug Targets, in *RSC Drug Discovery*, ed. B. M. Dunn, RSC Publishing, 2012, vol. 18.

10 C. M. Overall and O. Kleifeld, *Nat. Rev. Cancer*, 2006, **6**, 227–239.

11 B. Turk, *Nat. Rev. Drug Discovery*, 2006, **5**, 785–799.

12 X. S. Puente and C. López-Otín, *Genome Res.*, 2004, **14**, 609–622.

13 G. Murphy and H. Nagase, *Mol. Aspects Med.*, 2009, **29**, 290–308.

14 J. S. Ross and G. S. Ginsburg, *Am. J. Clin. Pathol.*, 2003, **119**, 26–36.

15 S. S. Kelkar and T. M. Reineke, *Bioconjugate Chem.*, 2011, **22**, 1879–1903. The special issue of September of *Acc. Chem. Res*. 2011 is devoted to Theranostics.

16 V. R. Devadasu, V. Bhardwaj and M. N. V. R. Kumar, *Chem. Rev.*, 2013, **113**, 1686–1735.

17 D. J. Lurie, D. M. Bussell, L. H. Bell and J. R. Mallard, *J. Magn. Reson.*, 1988, **76**, 366–370.

18 P. Mellet, P. Massot, G. Madelin, S. R. A. Marque, E. Harte, J.-M. Franconi and E. Thiaudière, *PLoS One*, 2009, **4**, e5244; P. Massot, E. Parzy, L. Pourtau, P. Mellet, S. Marque, J.-M. Franconi and E. Thiaudière, *Contrast Media Mol. Imaging*, 2012, **7**, 45–50; E. Parzy, V. Bouchaud, P. Massot, P. Voisin, N. Koonjoo, D. Moncelet, J.-M. Franconi, E. Thiaudière and P. Mellet, *PLoS One*, 2013, **8**(2), e57946.

19 T. Finkel and N. J. Holbrook, *Nature*, 2000, **408**(6809), 239–247.

20 M. B. Azad, Y. Q. Chen and S. B. Gibson, *Antioxid. Redox Signaling*, 2009, **11**, 777–790.

21 S. Reuter, S. C. Gupta, M. M. Chaturvedi and B. B. Aggarwal, *Free Radical Biol. Med.*, 2010, **49**, 1603–1616.

22 A. J. Montero and J. Jassem, *Drugs*, 2011, **71**, 1385–1396.

23 J. M. Matés, J. A. Segura, F. J. Alonso and J. Márquez, *Arch. Toxicol.*, 2012, **86**, 1649–1665.

24 P. Wardman, *Clin. Oncol.*, 2007, **19**, 397–417.

25 L. W. Jones and R. T. Major, *J. Am. Chem. Soc.*, 1927, **49**, 1527–1540.

26 G. A. Kovtun, A. L. Aleksandrov and V. A. Golubev, *Bull. Acad. Sci. SSSR, Ser. Chim.*, 1974, **10**, 2115–2121.

27 D. H. Solomon, E. Rizzardo and P. Cacioli, *Eur. Pat. Appl.* 135280, 1985; D. H. Solomon, E. Rizzardo and P. Cacioli, *US Patent* 4,581,429, 1986; *Chem. Abstr.*, 1985, **102**, 221335q.

28 D. Bertin, D. Gigmes, S. R. A. Marque and P. Tordo, *Chem. Soc. Rev.*, 2011, **40**, 2189–2198 and references cited therein.

29 P. Brémond and S. R. A. Marque, *Chem. Commun.*, 2011, **47**, 4291–4293; P. Brémond, A. Koïta, S. R. A. Marque, V. Pesce, V. Roubaud and D. Siri, *Org. Lett.*, 2012, **14**, 358–361; G. Audran, P. Brémond, S. R. A. Marque and G. Obame, *J. Org. Chem.*, 2012, **77**, 9634–9640.

30 J. C. Scaiano, T. J. Connolly, N. Mohtat and C. N. Pliva, *Can. J. Chem.*, 1997, **75**, 92–97.

31 Y. Kojima, S. Koda, H. Nomura and S. Kawaguchi, *Ultrason. Sonochem.*, 2001, **8**, 81–83.