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Synthesis, structure and EPR characterization of deuterated derivatives of Finland trityl radical

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Abstract

Substituted trityl radicals are important spin probes for functional electron paramagnetic resonance spectroscopy and imaging including oxygen and pH mapping *in vivo*. Here we report the synthetic procedure for large scale synthesis of deuterated Finland Trityl radical with superior EPR spectral properties and higher sensitivity towards oxygen concentrations in solution. Additionally Finland Trityl radicals substituted with linkers suitable for attaching peptide, or other synthetic precursors have been synthesized. The effect of deutero-substitution on EPR spectra of homologous derivatives has been evaluated. The compounds are potential candidates for targeted spin probes in EPR Imaging.

Triarylmethyl radicals, TAMs, and nitroxyl radicals, NRs, represent two main classes of soluble paramagnetic materials used for EPR spectroscopy and imaging applications. TAMs have advantages over NRs in extraordinary stability toward tissue redox processes, longer relaxation time and narrower line width making them particularly attractive for imaging applications[1]. However, undeveloped chemistry of the TAM radicals limits the number of available structures and their functional applications.

Triphenylmethyl radical was the first organic free radical synthesized by Gomberg more than hundred years ago[2]. Nevertheless, only recently the compounds with sterically protected trivalent carbon regained attention as the basic structural fragment for the synthesis of stable organic radicals. By the late 90s, Nycomed Innovation AB refined Gomberg's original trityl radical in order to avoid hydrogen hyperfine coupling and enhance its stability and water solubility[1, 3]. A new family of trityl spin probes, tetrathiatriarylmethyls, bearing four sulfur atoms on the phenyl ring was developed. The most representative members are TAM derivatives containing carboxyl group, namely cTAM, deuterated cTAM and more hydrophilic Oxo63 derivative (see Scheme 1).

The EPR spectra of these TAM derivatives[1, 4, 5] display a very narrow single line which is generally not broadened by interaction with proteins and other biological molecules, making them particularly attractive for imaging applications using EPR imaging, EPRI, and proton electron double resonance imaging, PEDRI (also known as Overhouser magnetic resonance imaging, OMRI)[6, 7]. In the latter case, the long relaxation time of TAMs makes them easily saturatable by radio frequency irradiation, and provides an advantage over NR for PEDRI

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applications by allowing enhancement of sensitivity and resolution with less heating of the sample[1, 8, 9]. Moreover, TAM radicals, due to their long relaxation time, are the superior probes for pulsed EPR/EPRI. Applications of TAM radicals include EPR oximetry[1, 8, 9], recently reported sensitivity to the superoxide radical anion[10, 11] and pH[12, 13] and their use as hyperpolarizing agents in dynamic nuclear polarization (DNP)-enhanced NMR[14] and MRI[15].

The extraordinary stability *in vivo*, very narrow single EPR line of about 100 mG or less, and oxygen-induced line broadening make TAMs the most efficient soluble oxygen probes. Oxygen-induced broadening of the TAMs in water is about (300–500) mG/mM of oxygen[1, 13] similar to that for the NRs. On the other hand, the concentration broadening of the TAMs is about 10–30 mG/mM[1] which is one order of magnitude less than that for the NRs[16]. These properties make TAM radicals superior oximetric probes for in vivo EPR, EPRI and PEDRI applications [1, 8].

The synthetic chemistry of TAM probes, while it is still in its infancy, is becoming a fast developing area of research. First reports on the synthesis of TAMs useful for EPRI and PEDRI have appeared in the patent literature[3]. The published procedures have been difficult to duplicate, and the large scale synthesis of these compounds has proven to be very challenging. Recently creative efforts have been done for the synthesis of these complex molecules[4, 5, 17]. We have published a large scale synthesis of the Finland trityl, cTAM, based on a few modifications of the original literature[4]. Fluorinated TAMs[18] possessing a high affinity to fluorous media were designed for assessment of tumor oxygenation using biocompatible perfluorocarbon emulsions. Recently we developed TAM structures with dual function pH and oxygen sensitivity [12, 13], and probes with enhanced sensitivity to oxygen due to doublet spectral pattern[19]. Dendritic[20] and ester[21] derivatives of Finland trityl were also reported as potentially valuable probes with enhanced stability and ability for intracellular delivery.

The isotopic substitution of the 36 methyl protons in the three aryl groups of TAMs for deuterons result in further significant decrease of their EPR linewidth which is important for the applications of TAMs as functional probes1. However, until now the synthesis of several deuterated TAMs were published only in patented literature[3]. Herein, we report the improved procedure for the synthesis of deuterated Finland trityl (cTAM*) and several new deuterated cTAM derivatives with the linkers attached to the carboxylic groups allowing for further functional modification. The effect of the isotopic substitution on the EPR spectra is described.

To synthesize cTAM* a slightly modified procedure previously developed in our group for large scale synthesis of cTAM radical was applied[4] (see Scheme 2).

The exchange of proton with deuterons observed during the first step was less than a few percent. The degree of deuteration of the product 2 in all preparations exceeded 95 % according to ^{1}H NMR data and was even larger (> 97 %) at larger scale synthesis.

An introduction of new groups and/or linkers in the TAM structure allows for adjustment of their functional properties, such as solubility, stability and sensitivity to oxygen and pH[13, 17, 18, 20]. The aTAM derivative with positively charged ammonium group and its deuterated analog, aTAM*, were synthesized as shown in the Scheme 3. The carboxylic moiety of cTAM was activated by standard peptode coupling conditions using O-(Benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate, HBTU, N,N-dimethylaminopyridine, DMAP, and triethylamine, TEA, followed by the addition of choline chloride.

An additional series of TAM_e derivatives based on glycolic acid ester, followed by addition of choline chloride was synthesized as shown in the Scheme 4. The compound $cTAM_e$ and its deuterated analog $cTAM_e$* with carboxylic end group, and $aTAM_e$ and $aTAM_e$* with

ammonium end group were obtained. The structures and EPR spectral parameters are summarized in Table 1 and detailed procedures and EPR spectra are available in the supporting information.

The EPR peak-to-peak linewidth, Δ_{pp}, of cTAM* was found to be 65 mG in anoxic solution in agreement with the previously reported data[1]. This 1.5 fold line narrowing compared with 95 mG linewidth of cTAM corresponds to more than two fold increase in spectral intensity, and provides significant advantage for EPR/EPRI applications.

Similar narrowing effect of deutero-substitution on the EPR linewidth was observed for aTAM, $cTAM_e$, and $aTAM_e$ (see Table 1 for the linewidth of the resolved line). However, additional hyperfine splitting (septet with peak intensity ratio 1:6:15:20:15:6:1) appears from 6 protons of methylene group, a_H, adjacent to aryl caboxylate. Interestingly, the a_H hyperfine splitting is almost twice larger for aTAM (110 mG) with positively charged ammonium group compared with $cTAM_e$ (60 mG) with carboxyl group in agreement with expected higher electron attracting inductive effect of the ammonium function. An increase of the linker length between aryl and ammonium groups in the compound $aTAM_e$ resulted in decrease of a_H hyperfine splitting from 110 mG to 70 mG. Note that the hyperfine structure from methylene protons was not resolved for the non-deuterated $cTAM_e$ and $aTAM_e$ derivatives with a_H values being significantly lower than the linewidth of the individual spectral components (see Table 1). For these compounds a_H values were first determined from the corresponding deuterated radicals and then used for calculation of a_H and Δ_{pp} values of non-deuterated compounds from their spectral simulation (see Fig.1 for the $aTAM_e$). The origin of multiplet spectral structure was further confirmed by the overnight incubation of $aTAM_e$* in CF_3COOD/D_2O solution, which resulted in partial substitution of the methylene protons for deuterons and corresponding disappearance of the multiplet structure (see Fig. 1c for the EPR spectrum of the $aTAM_e$**).

The EPR spectra of the cTAM and its synthesized derivatives show similar oxygen-induced line-broadening about 70 mG/20 % [O_2]. Among the deuterated cTAM derivatives the compounds cTAM*, $cTAM_e$* and $aTAM_e$** demonstrate single EPR line in anoxic solution with the narrowest line for cTAM*. The cTAM* radical, therefore, has an advantage in oxygen sensitivity and simplicity of the EPR spectrum. The multiplet character of the EPR spectra of aTAM* and $aTAM_e$* derivatives make them less attractive for EPR oximetric applications. Nevertheless, they have their own advantages compared with Finland trityl derivatives containing carboxylic groups. First, as it was previously shown[4] cTAM has tendency to aggregation associated with protonation of its carboxyl groups and facilitated in environments with low pH and low polarity, e.g. in the presence of biomembranes. On the other hand, aTAM derivatives show pH-independent solubility in aqueous solution in mM range of concentration. Apparently, the presence of positively charged ammonium group in the structure of aTAM derivatives prevents their aggregation and, therefore, makes them potentially less toxic. Second, in general the EPR oximetric probes with multiplet spectral pattern possess higher sensitivity to oxygen at low oxygen tension compared with single-line EPR probes due to the opportunity to follow changes in peak intensity ratio of partially resolved components rather than EPR linewidth[19, 22]. Recently we described the cTAM derivative with enhanced sensitivity to oxygen down to 1 mmHg due to its partially overlapped doublet EPR spectrum[19]. Therefore, aTAM* and $aTAM_e$* derivatives may have an advantage when accurate measurements of low oxygen concentrations are required, e.g. to locate area of hypoxia (≤ 15 mmHg 23) or even to detect a threshold of true anoxia (≤ 1.5 mmHg).

In summary, we describe the synthesis of several new TAM radicals. The deuterated Finland trityl, cTAM*, was synthesized according to a modified protocol. The synthesis is very efficient in multigram scale. $cTAM_e$ structures, which can be used for further derivatization such as in designing peptide-TAM bioconjugates have been also synthesized. The EPR characterization

of newly synthesized compounds should help in predicting the EPR hyperfine pattern of other derivatized TAMs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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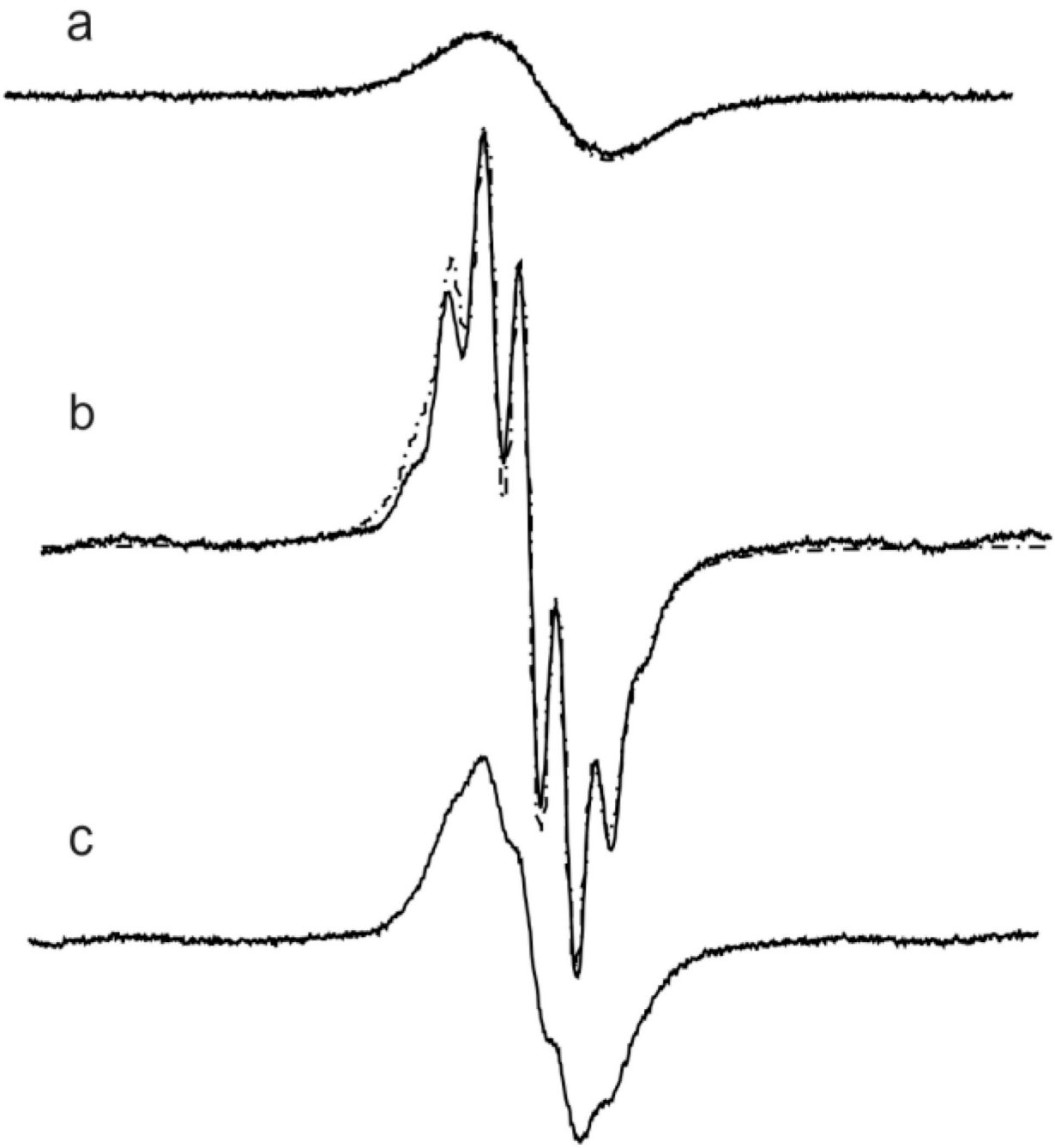
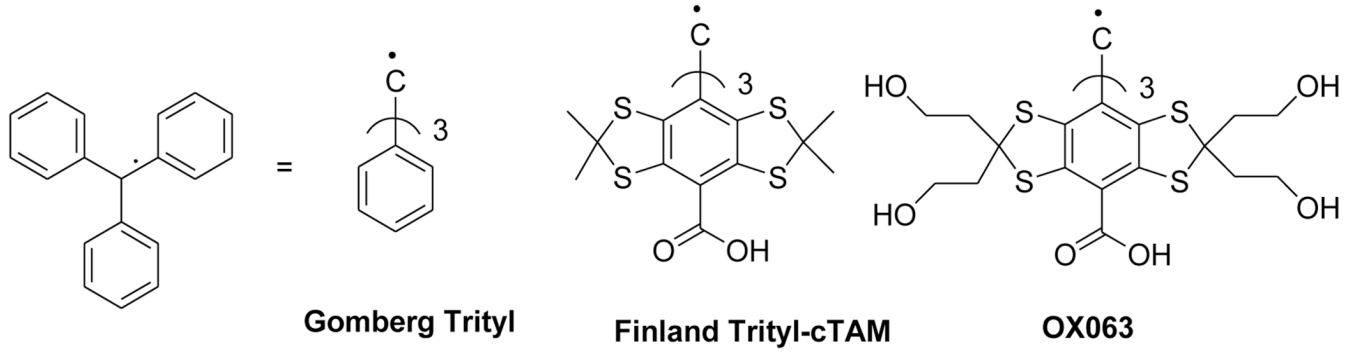
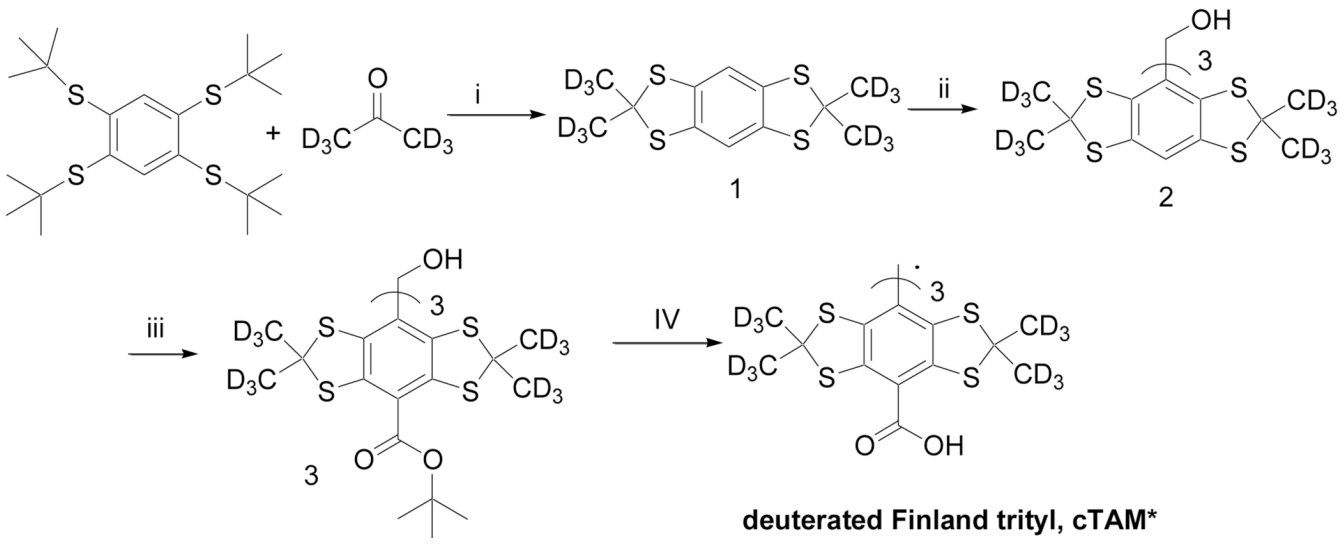


Figure 1.
X-band EPR spectra of 1 mM aTAMe (a), aTAMe* (b) and aTAMe** (c) measured in anoxic DMSO solutions radicals at room temperature. The spectra are shown with the same receiver gain. Spectral parameters were as follows: microwave power, 0.63 mW; modulation amplitude, 0.01 G; sweep width, 2G; number of points, 1024. The simulated EPR spectra (a) and (b) are shown by dotted lines.

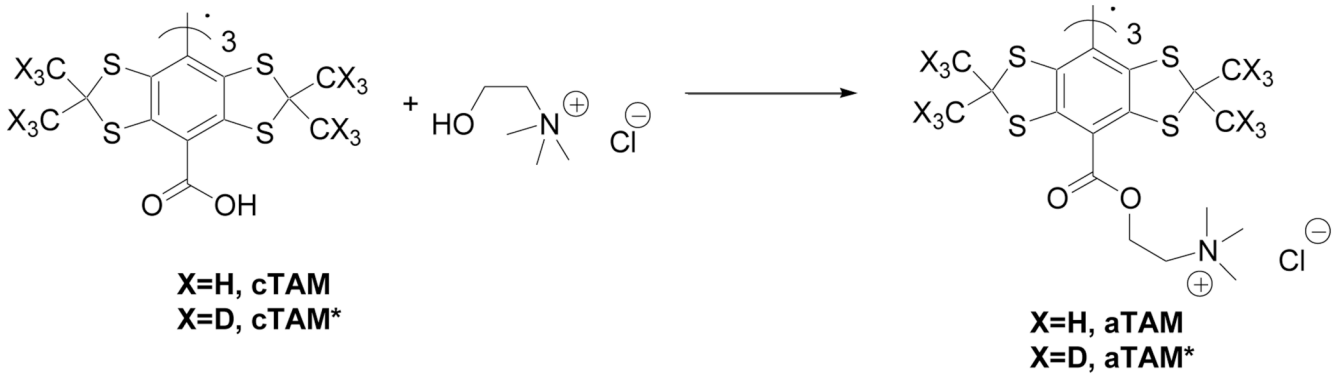


Scheme 1.
Representative structures of trityl radicals.

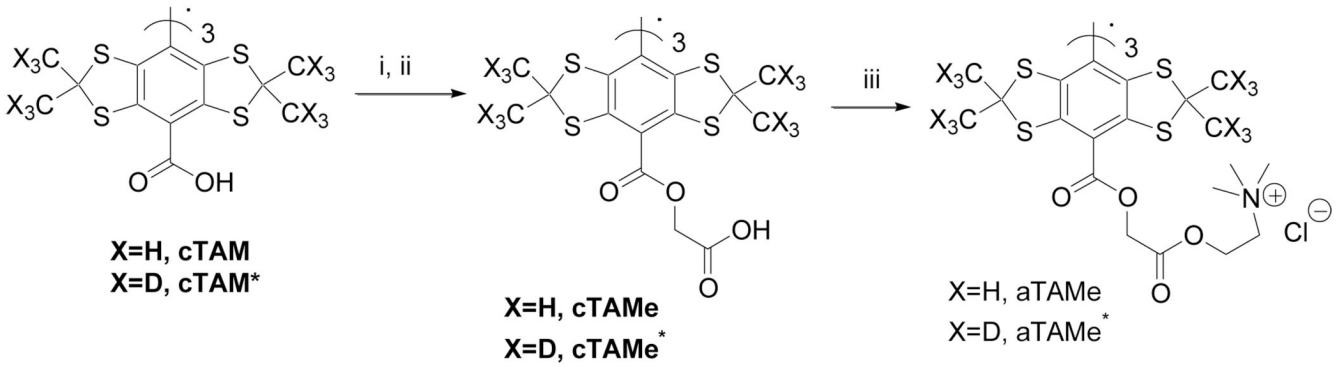


Scheme 2.

Synthesis of deuterated Finland trityl radical, cTAM*: (i) HBF_4, toluene, d6-acetone 70 %; (ii) n-BuLi, Et_2O; CH_3COCl, 70 %; (iii) n-BuLi, TMEDA, DiBoc, 44 %; (IV) CF_3COOH; 95%.



Scheme 3.
HBTU, DMAP, TEA, DMF, 65 %.



Scheme 4.

(i) HBTU, DMAP, TEA, DMF glycolic acid, tert-butylester; (ii) Trifluoroacetic acid 98 % over two steps; (iii) HBTU, DMAP, TEA, DMF, choline chloride; 60 %.

Table 1

EPR spectral parameters of the cTAM, aTAM, $aTAM_e$, $cTAM_e$ and their deuterated analogs obtained from EPR measurements in anoxic solutions.

TAM	Δ_{pp}, ±5 mG (resolved line)	a_H (CH_2), ±2 mG	Δ_{pp}^t, ±5 mG (unresolved multiplet)
cTAM	95	-	-
cTAM*	65	-	-
aTAM	92	110	-
aTAM*	60	110	-
$cTAM_e$	100	60	220
$cTAM_e$*	70	60	≈190*
$aTAM_e$	100	70	230
$aTAM_e$*	60	70	-

* the spectral line was only partially resolved