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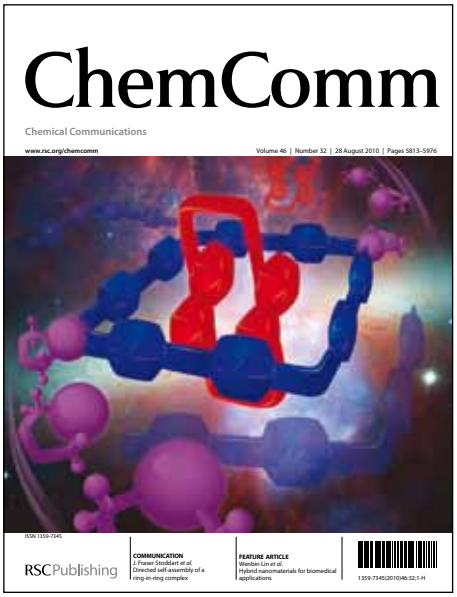

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ARTICLE TYPE

Fluoromethylated derivatives of carnitine biosynthesis intermediates – synthesis and applications

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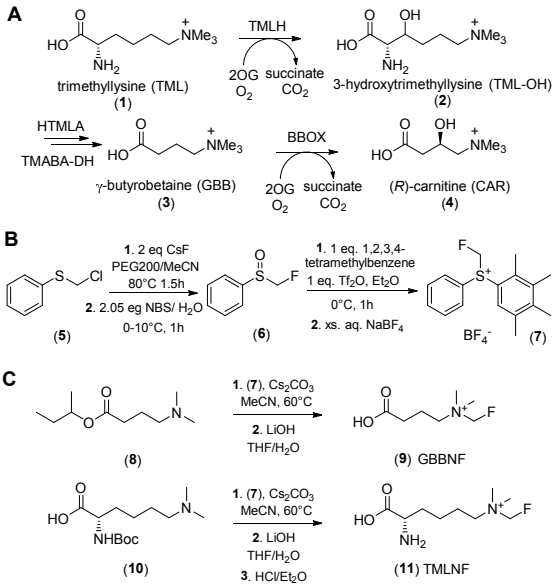
A convenient method for the synthesis of fluoromethylated carnitine biosynthesis intermediates, i.e. fluorinated derivatives of γ-butyrobetaine and trimethyllysine, is described. The fluoromethylated probes were useful in both *in vitro* and in cell based assays employing ^{19}F NMR and LC-MS analyses.

Carnitine, an essential metabolite in humans and other eukaryotes, is vital for the transport of long chain fatty acid into mitochondria[1]. In animals carnitine is biosynthetised from trimethyllysine (TML) in four enzyme-catalysed steps[2-4]. The first and last steps in the pathway are catalysed by trimethyllysine hydroxylase (TMLH) and γ-butyrobetaine hydroxylase (BBOX), both of which belong to the 2-oxoglutarate (2OG) dependent oxygenase superfamily. TMLH and BBOX catalyse hydroxylation of *N*$^{\varepsilon}$-trimethyllysine (TML, (**1**)) and γ-butyrobetaine (GBB, (**3**)) respectively (Scheme **1A**)[5,6]. The inhibition of human carnitine biosynthesis reduces fatty acid metabolism and has been targeted for the treatment of cardiovascular disease[7,8]. With respect to efforts aimed at understanding biological roles of 2OG dependent oxygenases in carnitine metabolism[9,10], we are interested in developing reagents that enable assays for carnitine biosynthesis enzyme activities, both *in vitro*[11] and in more biologically representative systems. Given that the monofluoromethyl group is similar in size to the methyl group, we envisioned fluoromethylated GBB (**9**) and TML (**11**) analogues as potentially useful tool compounds for monitoring carnitine biosynthesis.

Organofluorine compounds have substantial applications in chemistry and chemical biology; their applications include the modification of physicochemical and conformational properties of small molecules, and uses in radiomedicine as tracers[12] or labels for NMR studies[13,14]. ^{19}F NMR is emerging as a valuable tool for studying biochemical processes *in vitro* and *in vivo*[15]. The principle advantage of ^{19}F NMR is that the absence of endogenous fluorinated species in most living organisms eliminates problem of background interference and signal overlap. However, the use of ^{19}F NMR is often limited by the availability of appropriately labeled fluorinated compounds.

Various approaches for the incorporation of fluorine into small molecules are available, but reagents for electrophilic monofluoromethylation are limited[16]. Prakash *et al.* reported an air and moisture stable electrophilic fluoromethylation reagent (**7**)



Scheme 1 Synthesis of fluorinated analogues of carnitine biosynthesis metabolites. **A** – The carnitine biosynthesis pathway in mammals. **B** – Prepartaion of fluoromethylating agent (**7**). **C** – Synthesis of fluorinated derivative of γ-butyrobetaine (GBBNF, (**9**)) and trimethylysine (TMLNF, (**11**)). NBS – *N*-bromosuccinimide; Tf_2O – trifluoromethanesulfonic anhydride.

that reacts under mild conditions[17] with various nucleophiles, including amines[17,18]. The reported preparation of (**7**) required the use of liquefied CH_2FCl, which can be inconvenient. We therefore employed a route where chloromethylphenyl sulphide (**5**) was converted to the corresponding fluoride by reaction with caesium fluoride[19] and then directly oxidised with aqueous *N*-bromosuccinimide to yield fluoromethylsulphinyl benzene (**6**) – a known intermediate in synthesis of fluoromethylating reagent (**7**) (Scheme **1B**). We then used (**7**) to efficiently prepare *N*-CH_2F modified derivatives of GBB (**3**) and TML (**1**) in 2 or 3 steps, respectively, starting from appropriately protected *N,N*-dimethylated precursors (Scheme **1C**).

The fluoromethylated GBB analogue (GBBNF, (**9**)) was evaluated in reaction with purified recombinant human BBOX (hBBOX) *in vitro*. GBBNF (**9**) was found to be hBBOX substrate as demonstrated by ^{1}H NMR analyses (Fig. **S1**) undergoing hydroxylation at the C3 position to give fluoromethylated

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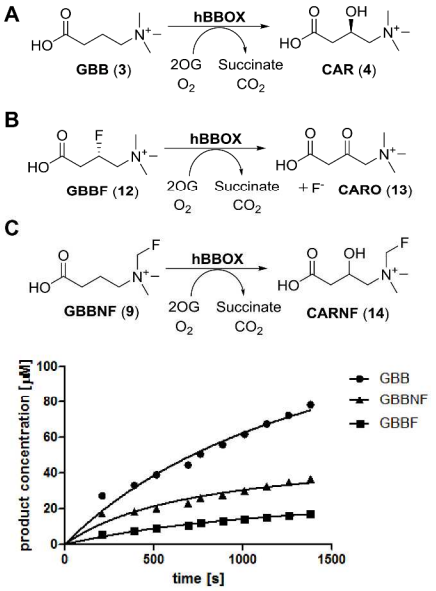

Figure 1 Reactions catalysed by BBOX. GBBNF is a BBOX substrate and undergoes hydroxylation in a position analogous to GBB hydroxylation. Time dependent product formation reveals that GBBNF is a better substrate than GBBF.

Table 1 Kinetic properties of fluorinated GBB analogues

		GBB (**3**)	GBBF (**12**)	GBBNF (**9**)
rate [μM/s]	Hydroxylation	0.123	0.027	0.083
	Succinate formation	0.162	0.063	0.097
Ratio of succinate formation to hydroxylation		1.1	2	1.3
Kinetic parameters	K_M [μM]	4.2	19.8	16.6
	k_{cat} [1/s]	0.83	0.14	0.30
	K_i [μM]	24.5	135	-

carnitine analogue (CARNF) (**14**) (Fig. **1C**; for NMR assignment of the product see Supporting Information – Fig. **S2**-**S4**), in an analogous manner to hBBOX catalysed GBB hydroxylation (Fig. **1A**). We have reported the synthesis of another fluorinated analogue of GBB – (*3S*)-fluoro-GBB (GBBF, (**12**)), C-3 hydroxylation of which leads to fluoride release concomitant with ketone (**13**) formation (Fig. **1B**)[11]. In NMR assays we did not observed fluoride release in reaction of GBBNF (**9**) with hBBOX, indicating that the fluoromethylated quaternary ammonium group is stable under the assay conditions. Comparison of the initial rates of hydroxylation of GBB (**3**), GBBNF (**9**) and GBBF (**12**) by hBBOX revealed that GBBNF (**9**) is a better substrate than GBBF (**12**) (Table **1**, Fig. **1**). The initial hydroxylation rate of GBBNF (**9**) is ~65% of the initial hydroxylation rate of GBB (**3**), while GBBF (**12**) is hydroxylated at ~20% of the initial rate of GBB (**3**). The K_M and k_{cat} values also reveal that the properties of GBBNF (**9**) as a substrate are closer to those of GBB (**3**) than GBBF (**12**) (Table **1**). Some 2OG dependent oxygenases catalyse turnover of 2OG independent of substrate transformation (uncoupled 2OG turnover)[5] which can manifest with a poor substrate[20]. We examined levels of uncoupled turnover when GBB (**3**) and its fluorinated analogues were used, by comparing rates of succinate formation to that of hydroxylation. The results showed GBBNF (**9**) to be similar to GBB (**3**) (ratio of succinate formation to hydroxylation 1.1 for GBB vs 1.3 for GBBNF),

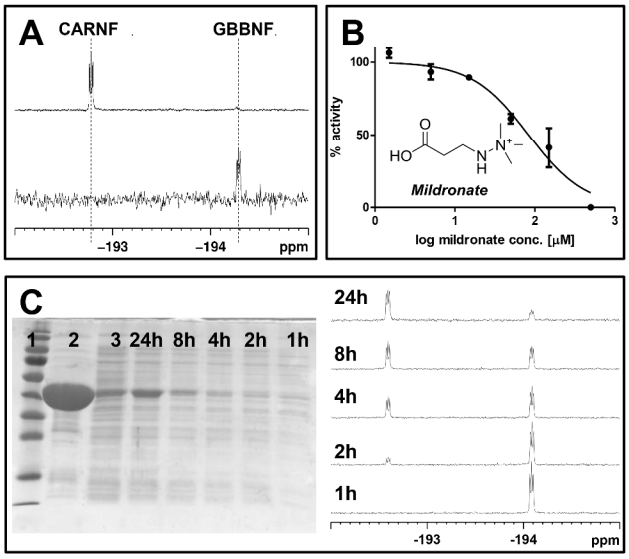

Figure 2 Applications of GBBNF (**9**). **A** – Conversion of GBBNF to CARNF by psBBOX can be followed by ^{19}F NMR. **B** – ^{19}F NMR based assay enables determination of dose-response curves for potential inhibitors, exemplified with the hBBOX inhibitor Mildronate. **C** – SDS-PAGE gel shows lysate samples from cells harvested at various times after IPTG induction (lanes 4-8). Molecular weight marker (lane 1), BBOX sample (lane 2) and uninduced cells harvested after 24h (lane 3) are shown. BBOX activity in cell lysates can be quantified by ^{19}F NMR.

while in case of GBBF (**12**), the ratio of uncoupled:coupled turnover was around two (Table **1**, Fig. **S5**). Note that, as for GBB (**3**), GBBF (**12**) displays substantial substrate inhibition (apparently K_i values in micromolar range – see Table **1**). Interestingly, the extent of substrate inhibition by GBBNF (**9**) was much less than for GBB (**3**) or GBBF (**12**) (Figure **S6**). The mechanism of substrate inhibition of hBBOX is unknown, but is of interest as it may be involved in regulating carnitine biosynthesis in cells.

hBBOX catalysed GBBNF (**9**) hydroxylation can be followed by ^{19}F NMR, because the fluorine shift of the product is distinctively different from a shift of the substrate (Fig. **2A**). In these compounds the ^{19}F resonance appears with a 1:1:1 triplet fine structure, not as a singlet. This structure arises from coupling of the fluorine to the adjacent quadrupolar ^{14}N nucleus (I= 1, 99.6% abundance) and is apparent because the highly symmetrical tetrahedral environment of the ^{14}N centre suppresses rapid quadrupolar relaxation that, in less symmetrical environments, leads to loss of coupling fine structure. ^{19}F NMR can also be employed for IC_{50} measurements (Fig. **2B**). We investigated hBBOX inhibition by Mildronate[7], which is an inhibitor and competitive substrate for hBBOX[9, 10]. The obtained IC_{50} value (82 μM) is similar to that obtained by ^1H NMR (34 μM) and fluoride release (65 μM) assays[11]. The differences likely reflect the difference in assay conditions used. Thus, ^{19}F NMR assay employing GBBNF (**9**) as a substrate is useful for *in vitro* activity studies. However, an advantage of the GBBNF (**9**) based system is its ability to monitor specific hBBOX turnover without the interference of any other non-fluorinated components, making it suitable for more biologically relevant assay conditions.

To test the turnover of fluorinated analogues in cells we used *E. coli* BL21 cells producing a prokaryotic BBOX homologue from

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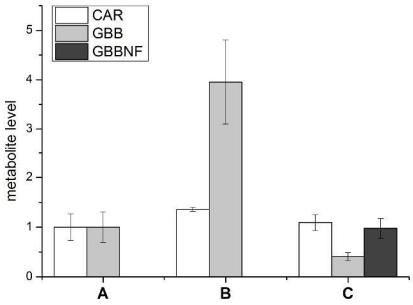

Figure 3 Levels of CAR (**4**), GBB (**3**) and GBBNF (**9**) in HEK 293T cells: A- control, B – treated with TML (**1**), C – treated with TMLNF (**11**). Error bars represent standard deviation of n=3 samples.

Pseudomonas sp. AK1 (psBBOX). We observed that GBBNF (**9**) is converted to CARNF (**14**) in crude cell lysates, providing 2OG was added to the reaction mixture and that turnover level is dependent on 2OG concentration (Fig. **S7**). The extent of GBBNF (**9**) turnover is dependent on the amount of psBBOX present in the extracts (Fig. **2C**, **S8**). Thus, ^{19}F NMR can be used to estimate psBBOX activities in cell lysates.

In addition to the use as a label for ^{19}F NMR studies, fluorine is a convenient marker for small molecules in MS based studies. We therefore investigated the metabolism of TMLNF (**11**) in human kidney cells (HEK 293T cells) using LC-MS. HEK 293T cells were grown either with or without TML (**1**) or TMLNF (**11**) added to the growth media. Cells were harvested, lysed and analysed for the carnitine related metabolites (Fig. **3**), using appropriate standards (Fig. **S10**). In none of the samples could TML (**1**) or TMLNF (**11**) be detected. In the sample treated with TML (**1**), elevated levels of GBB (**3**) were observed; indicating TML (**1**) penetrates cell membranes and is converted to GBB (**3**). Cells treated with TMLNF (**11**) contained lower amounts of GBB (**3**) than controls and substantial levels of GBBNF (**9**), which can only be formed from TMLNF (**11**), demonstrating TMLNF (**11**) is carried through the first enzyme catalysed step of carnitine biosynthesis. All of the samples contained similar levels of carnitine (within error), but no CARNF (**14**) was observed in the TMLNF (**11**) treated sample; this result may reflect the differences in affinities of GBB (**3**) and GBBNF (**11**) to hBBOX, as reflected in the *in vitro* by kinetic data (Table **1**).

In conclusion we have described an efficient procedure for the synthesis of the electrophilic monofluoromethylation reagent[17] (**7**) that enables convenient preparation of fluoromethylated quaternary ammonium derivatives, e.g. trimethyllysine and carnitine related compounds. The utility of these compounds for enzymatic assays was demonstrated employing ^{19}F NMR with recombinant hBBOX and crude cell lysates. LC-MS studies enabled tracking of fluorinated intermediates in human cells. Quaternary ammonium derivatives have widespread pharmaceutical and industrial applications e.g. as antimicrobial agents[21] or phase transfer catalysts in organic chemistry[22]. They are also ubiquitous class of metabolites, present in all living organisms[23]. In humans and animals *N*-methylated lysine, arginine and nucleic acid compounds play crucial roles in epigenetic regulation. We hope that the development of appropriate fluorinated small molecules will enable work aimed at understanding role of methylation in epigenetics.

Notes and references

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† Electronic Supplementary Information (ESI) available: synthesis procedures, assay conditions, NMR assignments. See DOI: 10.1039/b000000x/

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