ω-Azido fatty acids as probes to detect fatty acid biosynthesis, degradation, and modification[S]

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Abstract FAs play a central role in the metabolism of almost all known cellular life forms. Although GC-MS is regarded as a standard method for FA analysis, other methods, such as HPLC/MS, are nowadays widespread but are rarely applied to FA analysis. Here we present azido-FAs as probes that can be used to study FA biosynthesis (elongation, desaturation) or degradation (β-oxidation) upon their uptake, activation, and metabolic conversion. These azido-FAs are readily accessible by chemical synthesis and their metabolic products can be easily detected after click-chemistry based derivatization with high sensitivity by HPLC/MS, contributing a powerful tool to FA analysis, and hence, lipid analysis in general.—Pérez, A. J., and H. B. Bode. **ω-Azido fatty acids as probes to detect fatty acid biosynthesis, degradation, and modification. *J. Lipid Res.* 2014.** 55: **1897–1901.**

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FAs are found in all known living organisms, playing a vital role in cell compartmentalization, energy storage, and secondary metabolite production. In bacteria, most FAs are found in the cell membrane as part of the lipid bilayer (1). The actual FA profile of a cell can strongly vary depending on environmental and developmental conditions requiring de novo biosynthesis, degradation, and modification of the FAs involved (2–5). The monitoring of these metabolic pathways is usually conducted by GC-MS of FA methyl esters or other volatile FA derivatives (6). In spite of its merits, such as high sensibility and direct observability of especially volatile natural products, it is desirable to also have simple and effective methods of FA analysis via HPLC/MS, which today is a widespread tool in analytical chemistry as well. However, HPLC/MS-based FA analysis is difficult, mainly because of little ionizability and hence little signal strength. Small modifications introduced to certain FAs, however, can greatly increase ionizability while decreasing lipophilicity, which in turn increases signal resolution on reversed phase HPLC systems (7).

The method we developed for HPLC/MS-based detection of FA intermediates involves three steps: *i*) simple preparation of FAs with a terminal azido group (AFAs) that allows most FA modifications to occur; *ii*) use of these AFAs as metabolic probes and labeling of the in vivo formed derivatives in the corresponding organism with tetramethoxydibenzoazacyclooctyne (TDAC) (**1**, **Fig. 1A**), a cyclooctyne synthesized originally by Starke, Walther, and Pietzsch (8, 9); and *iii*) the detection of the clicked compounds by HPLC/MS. Although usually a reporter function such as a fluorophore is linked to the alkyne reagent, no such modifications were needed here, because the formation of an electron-rich triazole ring make it quite susceptible to protonation and thus detection by MS, especially as the molecular mass range of the clicked FAs differs strongly from other lipophilic compounds found in most cells. AFAs are bioorthogonal, do not react with other functional groups other than alkynes, are easily taken up by the cells like other FAs when externally added, and can be used to follow the fate of AFAs in a given organism in real time. Thus AFAs will add to the overall toolbox of lipid analysis in general. Similarily, alkyne-modified cholesterol has been used recently to trace cellular cholesterol metabolism and localization (10).

MATERIALS AND METHODS

General experimental procedures

Solvents and reagents were obtained from Sigma-Aldrich (München, Germany). TDAC was synthesized using the procedure described by Starke, Walther, and Pietzsch (9) and can be obtained from the authors of this work. Alternative cyclooctynes,

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Abbreviations: AFA, FA with a terminal azido group; EIC, extracted ion chromatogram; LB, Luria-Bertani; NAC, *N*-acetylcysteamine; TDAC, tetramethoxydibenzoazacyclooctyne.

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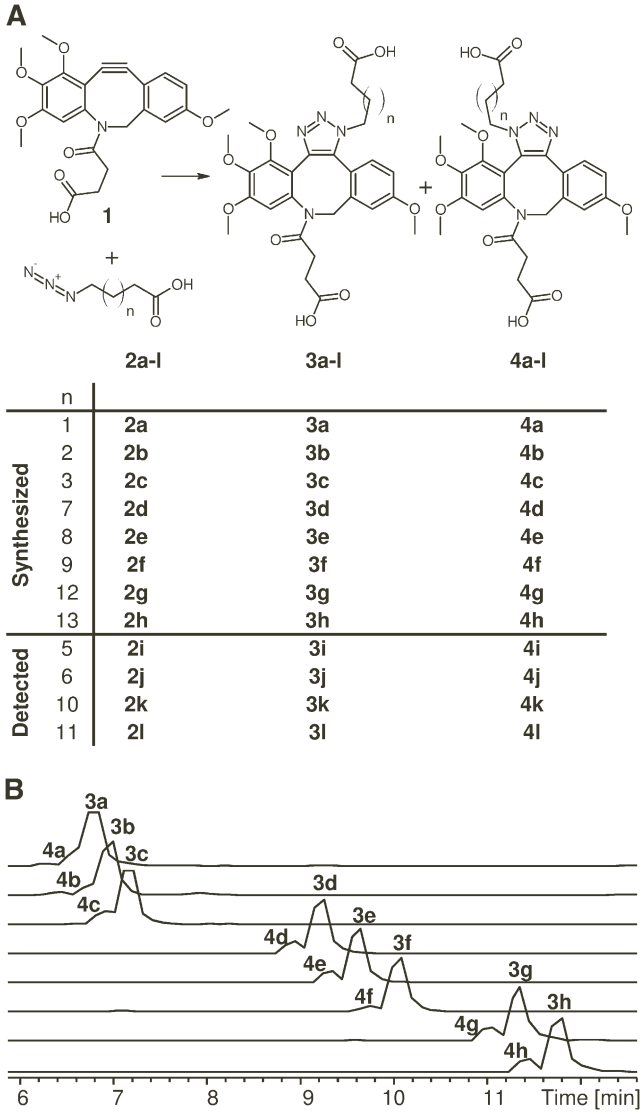

	n	2a-l	3a-l	4a-l
Synthesized	1	2a	3a	4a
	2	2b	3b	4b
	3	2c	3c	4c
	7	2d	3d	4d
	8	2e	3e	4e
	9	2f	3f	4f
	12	2g	3g	4g
	13	2h	3h	4h
Detected	5	2i	3i	4i
	6	2j	3j	4j
	10	2k	3k	4k
	11	2l	3l	4l

Fig. 1. A: Reaction of TDAC with various AFAs resulting in two regioisomers per AFA. Several AFAs (**2i–l**) were only produced in vivo and henceforth detected as their clicked products (**3i–l** and **4i–l**). B: EICs of the click product of TDAC with the eight synthesized AFAs. Where visible, the smaller peak arises from **4a–h** and the larger one from **3a–h**.

such as dibenzocyclooctyne-amine, with comparable structures can be obtained commercially from Sigma-Aldrich and mostly share the abilities of the cyclooctyne chosen in this work. Silica chromatographic purification was performed on a Biotage SP1™ Flash purification system (Biotage, Uppsala, Sweden), using 40+M, 25+M, or 12+M KP-Sil cartridges (Biotage, Uppsala, Sweden) in combination with an UV detector. ^{1}H, ^{1}H-^{1}H-COSY, ^{1}H-^{13}C-HSQC, and ^{1}H-^{13}C-HMBC NMR spectra for the synthesis products were recorded on a Bruker AV500 (500 MHz), AV400 (400 MHz), or AM250 (250 MHz) spectrometer using $CDCl_3$ or CD_3CN as solvent and internal standard, using the chemical shifts described by Gottlieb et al. (11) with the exception of the ^{13}C-shift of $CDCl_3$, which was set to 77.00 ppm. ^{1}H-NMR: $CHCl_3$, δ = 7.24 ppm; CH_3CN, δ = 1.96 ppm; ^{13}C-NMR: $CDCl_3$, δ = 77.00 ppm; CD_3CN, δ = 118.26 ppm. ESI HPLC/MS analysis was performed with a DionexUltiMate 3000 system coupled to a Bruker AmaZon X mass spectrometer and an Acquity UPLC BEH C18 1.7 μm RP column (Waters) using a MeCN/0.1% formic acid in water gradient ranging from 5 to 95% in 22 min at a flow rate of 0.6 ml/min (12). High resolution mass spectra were obtained from a MALDI LTQ Orbitrap XL (Thermo Fisher Scientific, Inc., Waltham, MA) equipped with a laser at 337 nm. A 4-chloro-α-cyanocinnamic acid matrix was used, and the sum formulas and according masses were internally calibrated using fluorescein (monoisotopic mass = 332.068473) as a standard (13).

Bacterial strains and culture conditions

All *Escherichia coli* strains used in this study were grown on solid and liquid Luria-Bertani (LB) (pH 7.0) medium at 30°C and 180 rpm on a rotary shaker. The mutants and controls obtained from the Coli Genetic Stock Center were grown in the presence of kanamycin (40 μg/ml). *Psychrobacter urativorans* (ATCC 15174) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) and grown on solid and liquid Oxoid nutrient broth (DSMZ medium 948) at 16°C on a rotary shaker at 210 rpm.

Feeding experiments for observation of FA degradation and desaturation

The feeding experiments for the observation of the degradation of FAs were conducted with 16-azidohexadecanoic acid. The compound was fed at a final concentration of 1 mmol/l to a culture of *fadE*, *fadA*, and *ilvE*, respectively. Cultures were grown in 250 ml Erlenmeyer flasks containing 30 ml of LB medium. Inoculation was performed from a preculture that was incubated overnight. The optical density was set to 0.1 and the culture was incubated at 30°C and 180 rpm for 3 h before feeding ensued and samples of 1 ml were taken. The samples were frozen in liquid nitrogen and freeze-dried before adding 1 ml 1 M NaOH and heated to 80°C for 4 h. Thereafter, the now clear solution was acidified with 190 μl of 6 M HCl and 1 ml of hexane was added. After vigorous stirring, the samples were centrifuged for 5 min at 4,000 rpm (3,220 *g*) and 700 μl of the supernatant were removed, dried, and taken up in 500 μl of acetonitrile, after which 40 μl of a 10 mM solution of the prepurified raw product of **1** was added. The reaction was allowed to occur at room temperature for 24 h before HPLC/MS analysis. The experiments with *P. urativorans* were conducted in the same fashion under different incubation conditions as described above.

Feeding experiments for FA elongation observation

The feeding experiments for the observation of the elongation of FAs were either conducted with *S*-(5-azidopentanoyl)-*N*-acetylcysteamine or *S*-(6-azidohexanoyl)-*N*-acetylcysteamine, respectively. The compounds were fed at a final concentration of 2 mmol/l to a culture of *fadE*, *fadA*, and DH10B. Cultures were grown in 100 ml Erlenmeyer flasks containing 10 ml of LB medium, including kanamycin in the concentration mentioned above in the case of *fadE* and *fadA*. Inoculation was performed from a preculture that was incubated overnight. The optical density was set to 0.1 and the culture was incubated at 30°C and 180 rpm for 2 h before feeding ensued and samples of 700 μl were taken. To these samples 200 μl 4 M NaOH were added, after which the mixture was heated to 90°C for 1 h. Thereafter, the now clear solution was acidified with 200 μl of 6 M HCl and 800 μl of hexane were added. After vigorous stirring, the samples were centrifuged for 5 min at 13,300 rpm (17,000 *g*) and 600 μl of the supernatant were removed, dried, and taken up in 100 μl of acetonitrile, after which 10 μl of a 0.1 M solution of the prepurified raw product of **1** was added. The reaction was allowed to occur at 50°C for 1 h before HPLC/MS analysis.

RESULTS AND DISCUSSION

Prior to the application of AFAs as molecular probes for FA metabolism, a library of eight AFAs of varying chain

length was synthesized (**2a–h**, Fig. 1A) from the corresponding ω-bromo-FAs in a one-step reaction (supplementary Scheme I). TDAC [obtained by a simple three step synthesis (supplementary Scheme II) with good click properties and formidable solubility in a wide range of solvents, as it is common to heterocyclic methoxylated cyclooctynes (14, 15)] was then reacted with these AFAs and subsequently analyzed by HPLC/MS (Fig. 1). Because 15-bromopentadecanoic acid was not commercially available, it was synthesized from pentadecanolide by hydrolysis and subsequent bromination of the resulting 15-hydroxypentadecanoic acid for direct use in the corresponding AFA synthesis (supplementary Scheme III). The results showed tremendous signal amplification by a factor of about 5,000 in the extracted ion chromatogram (EIC) of the clicked product as the $[M+H]^+$ ion in comparison to the free AFA (supplementary Fig. IA), along with clear differences in retention time according to the chain length of the corresponding AFA (Fig. 1B). Due to this strong increase in sensitivity resulting from the formation of the triazole unit, a relative quantification between different TDAC-modified AFAs also seems to be reasonable. Each signal consists of a characteristic double-peak, representing the two regioisomers formed in different amounts in accordance with the findings of Starke, Walther, and Pietzsch (9) for **3b**/**4b**. This regioselectivity corresponds to the different steric demand of each of the transition states (9). It was observed that the more abundant isomers, **3a–h**, had slightly higher retention times than the less abundant isomers, **4a–h**. Analysis of the MS^2 fragmentation pattern revealed distinct neutral losses for each of the two isomers. In particular, the minor isomer always features a dominant loss of water, followed by loss of the carboxylic group in the form of a dihydroxycarbene, whereas the main isomer predominantly shows only the loss of the latter group (supplementary Fig. IB). Thus, by comparing the characteristic fragmentation patterns, retention times, and peak shapes, simple identification of FA metabolites becomes possible.

In order to test whether AFAs can be used for the analysis of FA metabolism, β-oxidation was investigated initially. β-Oxidation is the primary FA degradation pathway in most organisms, consisting of desaturation, hydratization, oxidation, and finally thiolysis of the resulting CoA-bound 3-keto-FA residue, resulting in the formation of acetyl-CoA and an acyl-CoA residue shortened by two carbon atoms (supplementary Fig. II) (16, 17). In most bacteria, the basic steps are performed by only three proteins: FadE (desaturation), FadB (hydratization and oxidation), and FadA (thiolysis) (18). Thus, a feeding experiment was conducted in which a long chain AFA was fed to *E. coli* DH10B. Hydrolysis of this culture and derivatization with TDAC allowed the combined detection of free and (previously) bound FA β-oxidation degradation products, clearly showing that ω-azido FAs are tolerated by the β-oxidation machinery. Indeed, C_{16}-AFA (**2h**) was readily degraded in C_2-steps, leading to a degradation product as short as 4-azidobutanoic acid (**2a**) resulting in the detection of **3a**/**4a** (**Fig. 2A**). Additionally, the 3-keto and the 3-hydroxy forms were also detectable when high concentrations of the corresponding nonoxidized AFAs were reached (supplementary Fig. III). The retention times of these metabolic intermediary products proved to be similar to their saturated counterparts for long chain AFAs, while being slightly lower for short chained AFAs and clearly different from the retention times of C_{n+1}-AFAs, which have the same nominal mass as the corresponding C_{n-3}-keto-AFAs.

Next, the degradation of **2h** was analyzed over time in different *E. coli* strains with mutations in *fadA* (JW5578) and *fadE* (JW5020), with *ilvE* (JW5606) as a control. The *fadA* mutant supposedly lacked the ability to conduct the final thiolysis step, whereas the *fadE* mutant lacked the acyl-CoA dehydrogenase. In the control strain (with a defect in leucine degradation not influencing FA metabolism), signals correlated to C_{14}- and C_{12}-AFA (**2l** and **2f**, respectively) showed a maximum at 2.3 and 2.8 h after the addition of **2h**, respectively, and also shorter degradation products could be observed (Fig. 2B). Notably, analysis of the *fadA* mutant showed no difference to the *ilvE* control strain (not shown), indicating the functional complementation of the *fadA* mutation, while the *fadE* mutant showed

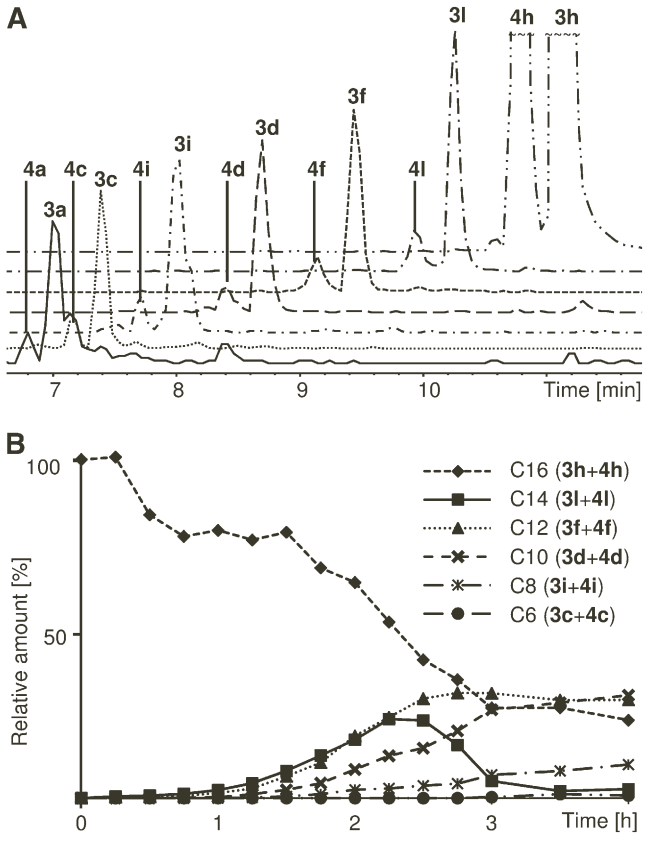

Fig. 2. A: EICs of click-labeled degradation products of **2h** 12 h after initial feeding at 1 mM. The same amount of **2h** was fed again right before the sample was taken. **3h**/**4h** = TDAC-C_{16}-AFA, **3l**/**4l** = TDAC-C_{14}-AFA, **3f**/**4f** = TDAC-C_{12}-AFA, **3d**/**4d** = TDAC-C_{10}-AFA, **3i**/**4i** = TDAC-C_8-AFA, **3c**/**4c** = TDAC-C_6-AFA, **3a**/**4a** = TDAC-C_4-AFA. B: Relative peak area (in percent relative to the amount of **3h**+**4h**) of **2h** degradation products when fed to *ilvE* mutant. Increasing amounts of C_{14}- and C_{12}-AFA can be seen 1 h after feeding, followed by further degradation products.

no degradation activity at all (supplementary Fig. IV) (19). The decrease in concentration of **2h** in the latter experiment might be attributed to absorption on the glass wall of the cultivation flasks or micelle formation in the medium. Similarly, C_{15}-AFA (**2g**) was also fed to the *E. coli* wild-type, and degradation products of uneven carbon chain length could be observed as expected (supplementary Fig. V). This also proved that AFA incorporation and degradation is not dependent on a specific chain length, but occurs with several long chain AFAs, furthermore indicating the broad applicability of AFAs for degradation studies.

Next, FA biosynthesis was studied, generally requiring the condensation of a given FA thioester with malonyl-ACP leading to an elongation in C_2-steps in a fashion reverse to FA degradation (supplementary Fig. II). Therefore, acyl-ACP-mimicking *S*-azidoacyl-*N*-acetylcysteamines (azidoacyl-NACs) of five or six carbons in acyl chain length were synthesized (**5a** and **5b**, respectively; see supporting information) and fed to the *E. coli fadE* mutant and to the wild-type (DH10B) as control. The wild-type showed only trace amounts of **3g** and **3k** (**Fig. 3A**), whereas elongated AFA derivatives up to C_{15}-AFA (**3g**) could be observed in the *fadE* mutant during the first 12 h after the addition of **5a** (Fig. 3B). No elongation could be observed when C_6-azidoacyl-NAC (**5b**) was fed (not shown), which implies that **5a** more closely resembles the natural FA and is well-tolerated by the FA biosynthesis machinery.

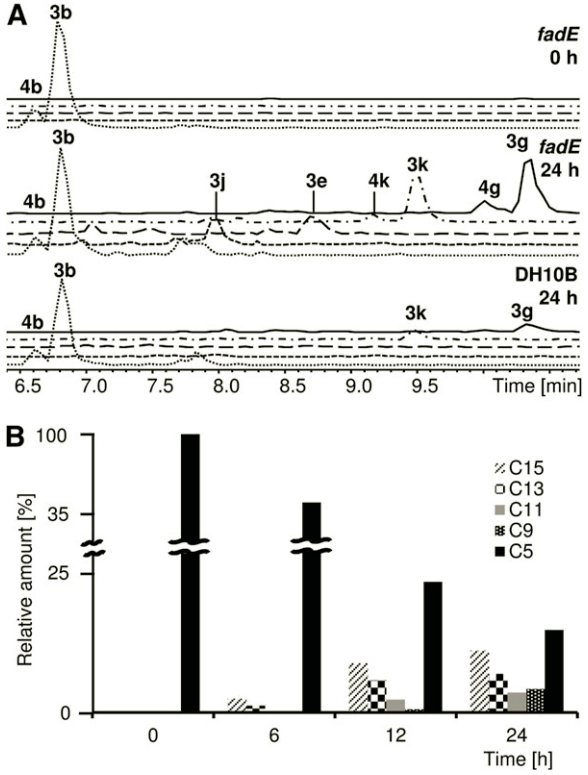

Fig. 3. A: EICs of clicked uneven AFAs immediately after and 24 h after feeding of **5a** (detected as **3b**/**4a**) to *fadE* mutant. The other EICs prove the production of C_9-AFA (**3j**), C_{11}-AFA (**3e**), C_{13}-AFA (**3k**/**4k**), and C_{15}-AFA (**3g**/**4g**). Even after 24 h the wild-type (DH10B) only shows minimal production of C_{13}-AFA (**3k**) and C_{15}-AFA (**3g**). B: Relative peak areas (in percent relative to the area of **3b**+**4b**) of various AFAs from the feeding experiment with the *fadE* mutant. C_{15}-AFA (as click product **3g**) can clearly be seen as emerging main product of FA elongation. Production of C_7-AFA could not be detected due to signal overlap with an impurity in the TDAC reagent.

Besides FA degradation and biosynthesis, FA modification can also be studied using the described approach: *P. urativorans*, formerly known as *Micrococcus cryophilus*, is a Gram-negative psychrophilic bacterium found in cold habitats that possesses a simple membrane FA composition, consisting mostly of Δ9-unsaturated palmitoleate and oleate residues (20, 21). When adapting to temperature changes in the range of 0–20°C, *P. urativorans* changes its FA profile by varying the ratio of the two main FA residues in the membrane (22, 23). Feeding C_{16}-AFA (**2g**) to a culture of *P. urativorans* at 16°C followed by the subsequent detection of a mono-unsaturated FA with slightly reduced retention time (**3m**/**4m**, **Fig. 4**) confirms that a desaturase is indeed involved in this process.

SUMMARY

The use of AFAs in combination with modern and easy to synthesize cyclooctynes, such as TDAC, has shown to be a viable method for the analysis of FA metabolism, ranging from biosynthesis to degradation and desaturation. Because no degradation products smaller than 4-azidobutanoic acid (**2a**) have been detected, it can be assumed that **2a** cannot be degraded. This might lead to increased availability of labeled FAs, even after prolonged metabolization, as compared with completely degradable isotope labels (24). The azido label forms an easily detectable triazole ring after reacting with a cyclooctyne, and is readily introduced into the desired target compound, thus allowing a wide range of applications in a time-resolved manner at low cost and high sensitivity. Our method can also easily be applied to study FA uptake or FA activation, which can be coupled to FA β-oxidation and can be detected by our approach. Moreover, it can not only be applied to FAs, but it can also be applied to lipid metabolism in general. For the latter, simply leaving out the hydrolytic step during sample preparation would enable the detection of acylcarnitines or CoA derivatives, as well as other lipid species carrying the respective AFA, not only in bacteria but also in all

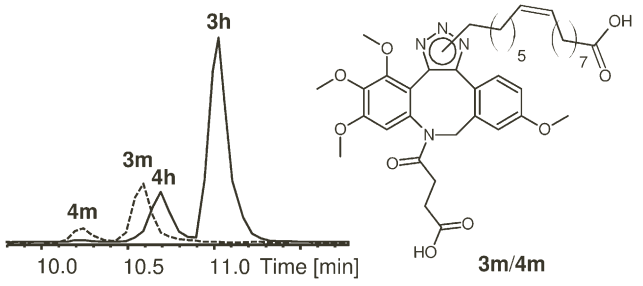

Fig. 4. Desaturase activity shown in *P. urativorans*. The EIC of the clicked AFAs shows that C_{16}-AFA (shown as click products **3h** and **4h**) is converted to the monodesaturated $C_{16:1}$-AFA (shown as click products **3m** and **4m**, with the regiochemistry assumed to be the same as with **3a–l** and **4a–l**, respectively).

organisms, including mammals, in which lipid metabolism needs to be studied.

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