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Mechanism of anaerobic degradation of triethanolamine by a homoacetogenic bacterium ☆

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Dedicated to Prof. Paolo Manitto on occasion of his 70th birthday.

Abstract

Triethanolamine (TEA) is converted into acetate and ammonia by a strictly anaerobic, gram-positive *Acetobacterium* strain LuTria3. Fermentation experiments with resting cell suspensions and specifically deuterated substrates indicate that in the acetate molecule the carboxylate and the methyl groups correspond to the alcoholic function and to its adjacent methylene group, respectively, of the 2-hydroxyethyl unit of TEA. A 1,2 shift of a hydrogen (deuterium) atom from $-CH_2-O-$ to $=N-CH_2-$ without exchange with the medium was observed. This fact gives evidence that a radical mechanism occurs involving the enzyme and/or coenzyme molecule as a hydrogen carrier. Such a biodegradation appears analogous to the conversion of 2-phenoxyethanol into acetate mediated by another strain of the anaerobic homoacetogenic bacterium *Acetobacterium*.

Keywords: Triethanolamine; Diethanolamine; Ethanolamine; Homoacetogenic bacterium; Anaerobic biodegradation; Radical mechanism

Triethanolamine (TEA: 2,2′,2″-nitrilotriethanol, **1**) is an industrially important chemical with an annual production exceeding 100,000 metric tons in the US [1]. It is used in the manufacture of a wide range of consumer products including household detergents, cosmetic and pharmaceutical preparations as well as in the formulation of aquatic herbicides, demulsifiers, cement additives, and metal cutting fluids [2]. Diethanolamine (DEA: 2,2′-iminodiethanol, **2**) is produced in a similar amount and for analogous purposes [3]. Therefore there is a need to understand the fate and the biodegradation pathway(s) of such xenobiotic substances. Although the aerobic microbial degradation of ethanolamines [4–13] and the enzymatic (anaerobic) B_{12}-dependent deamination of ethanolamine (EA: 2-aminoethanol, **3**) [14] have been investigated in considerable detail, little is known about the anaerobic fermentation of TEA [15] and DEA [3].

In 1994 Frings et al. [15], reported that a homoacetogenic bacterium isolated from sewage sludge (*Acetobacterium* sp., strain LuTria3) was able to ferment triethanolamine to acetate and ammonia according to the pathway of Fig. 1a. It must be noticed that 1/4 mol of acetate is synthesized from CO_2 through the acetogenic activity of the microorganism for each mol of acetate arising from the biodegradation of TEA.

A radical mechanism, as schematized in Fig. 1c, i.e., based on the intramolecular shift of the OH group, was suggested [15] by analogy with the rearrangement occurring in the diol dehydratase reaction. In this paper evidence is presented that all three ethanolamines (TEA, DEA, and EA) are processed by the bacterium LuTria3 to give acetate

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☆ *Abbreviations:* NMR, nuclear magnetic resonance; ESI-MS, electrospray ionization mass spectrometry; FAB-MS, fast atom bombardment mass spectrometry; TLC, thin layer chromatography; DMF, *N*,*N*-dimethylformamide; THF, tetrahydrofuran.

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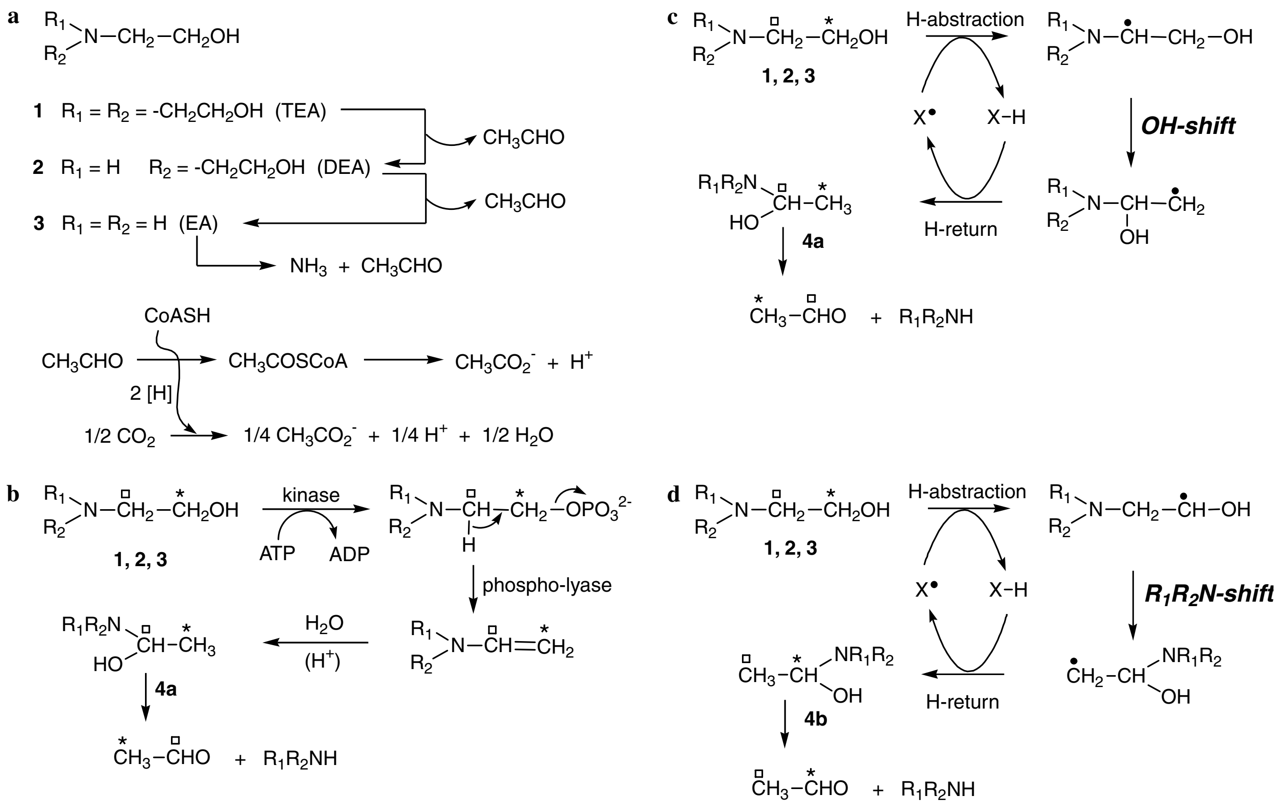

Fig. 1. Schematic deamination routes of ethanolamines to give acetaldehyde (or further oxidized products).

according to the mechanism of Fig. 1d, which is centered on the migration of the R_1R_2N group as a key step.

Materials and methods

General methods. TLC was performed on silica gel F_{254} precoated aluminum sheets (0.2 mm layer, Merck, Darmstadt, Germany); visualization was achieved by using a UV lamp or permanganate staining. Silica gel 60, 63–200 and 40–63 µm (Merck) was used for column and flash chromatography, respectively. NMR spectra were recorded on a Bruker AVANCE 400 Spectrometer using a XWIN-NMR software package and on a Bruker AC 300 instrument equipped with an ASPECT 3000 data system (Bruker, Karlsruhe, Germany). Chemical shifts (δ) are given in ppm and were referenced to the solvent signals (δ_H 7.25, δ_C 77.00; δ_H 2.50, and δ_C 39.50 ppm from TMS (tetramethylsilane) for $CDCl_3$ and DMSO-d_6, respectively) or to 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (δ_{Me} 0) in the case of D_2O/NaOD (pH > 10) solutions. ^{13}C NMR signal multiplicities were based on APT (attached proton test) spectra. Fast atom bombardment mass spectra (FAB-MS) were run on a VG 7070 EQ mass spectrometer and electrospray ionization mass spectra (ESI-MS) on a ThermoFinnigan LCQ Advantage spectrometer.

All reagents were of commercial quality or purified prior to use by standard methods. Acetic acid-d_4, nitrilotriacetic acid, diethyl iminodiacetate, glycine-2,2-d_2, and glycine methyl ester hydrochloride were from Aldrich.

*2,2′,2″-Nitrilotri(2,2′,2″-$^2H_6$)ethanol (**10**).* Benzyl bromoacetate-2,2-d_2 (**6**) was synthesized from commercially available acetic acid-d_4 (99.9 atom % D) (**5**) in 87% yield by the method described by Ochiai et al. [16]. ^1H NMR (400 MHz, $CDCl_3$) δ 5.23 (s, 2H, CH_2), 7.38 (m, 5H, aromatic H); ^2H NMR (61.4 MHz, $CHCl_3$) δ 3.89 ($BrCD_2$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 26.02 (quintet, J 23.7 Hz, $BrCD_2$), 68.49 (OCH_2), 128.98, 129.21,129.27, 135.63 (aromatic C), 167.65 (COO).

Commercially available glycine-2,2-d_2 (98 atom% D) (**7**) was converted into its ethyl ester hydrochloride [17] which was treated with aqueous 40% K_2CO_3 [18] to give **8** in 65% overall yield.

To a stirred suspension of benzyl bromoacetate-2,2-d_2 (**6**, 1.0 g, 4.3 mmol) and $KHCO_3$ (500 mg, 5.0 mmol) in dry DMF (6 ml) at 0 °C, **8** (180 mg, 1.7 mmol) dissolved in DMF (1 ml) was added over a 10-min period. The reaction mixture was stirred at 0 °C under nitrogen atmosphere for 30 min and then for 20 h at room temperature after which time it was diluted with diethyl ether (10 ml). The precipitate was filtered off, the filtrate washed with water (2 × 10 ml), and the combined aqueous washes were extracted again with diethyl ether (10 ml). The combined ether extracts were dried over Na_2SO_4 and evaporated under reduced pressure to give a crude product which was purified by flash chromatography eluting with n-hexane–ethyl acetate, 3:1 to give the triester (**9**) as an oil (475 mg, 69% yield). TLC, R_f 0.47, eluent as above; ^1H NMR (400 MHz, $CDCl_3$) δ 1.26 (t, 3H, J 7.2 Hz, CH_3), 4.17 (q, 2H, J 7.2 Hz, CH_2CH_3), 5.16 (s, 4H, CH_2Ph), 7.36 (m, 10H, aromatic H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.21 (CH_3), 54.45 (quintet, J 20.5 Hz, CD_2), 60.71 (CH_2CH_3), 66.45 (CH_2Ph), 127.64, 128.35, 128.60, 135.59 (aromatic C), 170.75, 170.66 (COO); ESI-MS m/z 428 $[M + Na]^+$.

The triester **9** (320 mg, 0.8 mmol) was dissolved in dry THF (5 ml) and added dropwise to a stirred suspension of $LiAlH_4$ (220 mg, 5.8 mmol) in dry THF (15 ml) at 5 °C under nitrogen atmosphere. Upon complete addition, the mixture was heated under reflux for 4 h and then stirred at room temperature overnight. After cooling to 0 °C, the reaction was quenched by the dropwise addition of saturated Na_2SO_4 solution until a white solid was formed. The resulting mixture was stirred for a further 30 min and then vacuum filtered. The filtrate was concentrated under vacuum to give a yellow oil (33 mg, 27%), while the precipitate was subjected to a Soxhlet extraction with THF for 20 h. The solvent was removed under vacuum and the resulting yellow-brown viscous oil was combined with the previous one and purified by column chromatography (MeOH-28% NH_3, 30:1) to give triethanolamine-2,2′,2″-d_6 (**10**) as a pale yellow oil (82 mg, 66% overall yield); TLC, R_f 0.52, eluent as above; ^1H NMR

(400 MHz, $CDCl_3$) δ 3.64 (OCH_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ 56.17 (NCD_2, quintet, J 21.3 Hz), 59.72 (OCH_2); ESI-MS m/z 156 $[M+H]^+$.

*2,2′,2″-Nitrilotri(1,1′,1″,2,2′, 2″-$^2H_{12}$)ethanol (**11**).* When the triester **9** was reduced with $LiAlD_4$, completely deuterated TEA **11** was obtained; ESI-MS m/z 162 $[M+H]^+$.

*2,2′,2″-Nitrilotri(1,1′,1″-$^2H_6$)ethanol (**14**).* Triethyl nitrilotriacetate (**13**, 300 mg, 1.1 mmol), prepared from nitrilotriacetic acid (**12**) as reported by Mo et al. [19], was reduced with $LiAlD_4$ (330 mg, 7.9 mmol) in THF (25 ml) as described above for the triester **9** to give **14** in 70% overall yield; ^1H NMR (300 MHz, DMSO-d_6) δ 2.56 (NCH_2); ^2H NMR (61.4 MHz, DMSO) δ 3.40 (OCD_2); ^{13}C NMR (75 MHz, DMSO-d_6) δ 57.05 (NCH_2), 58.22 (quintet, J 21.3 Hz, OCD_2); ESI-MS m/z 156 $[M+H]^+$.

*2,2′-Iminodi(1,1′-$^2H_4$)ethanol (**16**).* It was prepared by reduction of diethyl iminodiacetate (**15**) with $LiAlD_4$ using the procedure described by Ludeman et al. [20]. ^1H NMR (400 MHz, $CDCl_3$) δ 2.79 (NCH_2); ^2H NMR (61.4 MHz, $CHCl_3$) δ 3.64 (OCD_2); ^{13}C NMR (75 MHz, $CDCl_3$) δ 50.95 (NCH_2), 60.06 (quintet, J 21.4 Hz, OCD_2); ESI-MS m/z 110 $[M+H]^+$.

*(1-$^2H_2$)Aminoethanol (**18**).* It was prepared by reduction of glycine methyl ester hydrochloride (**17**) with $LiAlD_4$ in THF according to Schaffrath et al. [21].

Medium and growth conditions. Acetobacterium sp. strain LuTria 3 (DSM 8909) was grown at 28 °C in the dark, in CO_2/bicarbonate-buffered (30 mM, pH 7.2), sulfide-reduced (1 mM) freshwater mineral medium with 5 mM triethanolamine as sole organic carbon substrate under a N_2/CO_2 atmosphere (80:20 v/v) as previously described [15]. Triethanolamine was added from anoxic filter-sterilized stock solutions. Besides other vitamins the medium contained about 40 nM cyanocobalamin. Cells were grown as batch cultures of 0.5 or 1 L volume, in infusion bottles sealed with butyl rubber septa.

Cell suspension experiments with labeled triethanolamine. Cell suspensions were prepared under strictly anoxic conditions in an anoxic chamber as described in detail before [15]. The protein content in the cell suspension varied between 0.1 and 0.45 mg/ml. Cell suspensions were incubated with 4–5 mM labeled substrate, and substrate turnover was checked via acetate measurement by HPLC analysis. The supernatant was filtered through a cellulose acetate membrane filter with a pore size of 0.2 µm, and stored at 4 °C.

Isolation of acetate from the reaction mixture. The filtrate was alkalinized to pH 12 with 10 M NaOH, and evaporated to about one half its original volume in the vacuum at 60–70 °C. The alkaline solution was extracted three times with diethyl ether to remove remnant triethanolamine and other organics, before acidification to pH 1–2 with concentrated HCl. Then some NaCl was added to the aqueous phase and the acetic acid was extracted four times with diethyl ether, with a 5:1 ether-to-water volume ratio. After addition of 0.5 ml 2 M NaOH, the ether phase was concentrated to few milliliters *in vacuo*, and the product freeze dried.

Data of isolated sodium acetate samples. $CDH_2COO^-Na^+/CH_3COO^-Na^+$: ^1H NMR (400 MHz, D_2O) δ 1.939 (s, CH_3), 1.926 (triplet, $^2J_{HD} = 2.01$ Hz, $CDH2$); ^{13}C NMR (100 MHz, D_2O) δ 23.849 (s, CH_3), 23.617 (triplet, $J_{CD} = 19.5$ Hz, CDH_2), 181.985 (COO^-); FAB-MS m/z 105 and 106 $[M+Na]^+$. $CD_2HCOO^-Na^+/CH_3COO^-Na^+$: ^1H NMR (400 MHz, D_2O) δ 1.920 (s, CH_3), 1.893 (quintet, $^2J_{HD} = 2.06$ Hz, CD_2H); ^{13}C NMR (100 MHz, D_2O) δ 23.607 (s, CH_3), 23.151 (quintet, $J_{CD} = 18.9$ Hz, CD_2H), 181.935 (COO^-); FAB-MS m/z 105 and 107 $[M+Na]^+$. $CD_3COO^-Na^+/CH_3COO^-Na^+$: ^2H NMR (61.4 MHz, H_2O) 1.821 (s, CD_3); FAB-MS m/z 105 and 108 $[M+Na]^+$.

Results and discussion

To distinguish between the two possible fates of the carbon atoms of the C2-unit of TEA at the end of its complete degradation into acetate by the *Acetobacterium* strain LuTria3 [15], i.e., the conversion of the $-CH_2OH$ group into either $-CH_3$ or $-COOH$, specifically deuterated triethanolamines **10** and **14** were synthesized as shown in Figs. 2a and b and submitted to fermentation. While the preparation of **14** by $LiAlD_4$ reduction of **13** (obtained

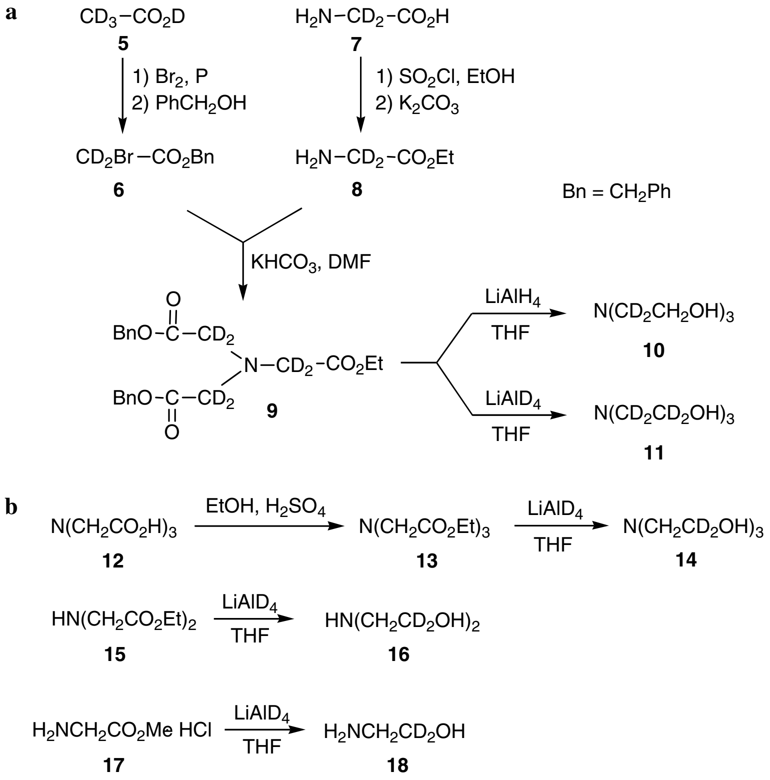

Fig. 2. Synthesis of deuterium-labeled substrates.

from commercial nitrilotriacetic acid **12**) was straightforward, a convergent synthesis starting from **5** and **7** was developed to produce compound **10**.

After the complete fermentation of 2,2′,2″-nitrilotri(2,2′,2″-$^2H_6$)ethanol (**10**, D_6-molecules >94%) by *Acetobacterium* under a N_2/CO_2 atmosphere, sodium acetate was isolated from the culture supernatant and examined by ^1H and ^{13}C NMR spectroscopy. The methyl region of these spectra showed peaks assignable to a mixture of di- and non-deuterated acetate molecules only. The former exhibited the typical pattern of signals due to CHD_2, i.e., a 1:2:3:2:1 quintet [22] (Fig. 3a) centered upfield with respect to the non-deuterated methyl group ($^2J_{HD} = 2.06$ Hz; isotope shift, $^2\Delta H(D_2) = 27.0$ ppb) [23–25]. The expected signals, due to a dideuterated methyl group ($J_{CD} = 18.9$ Hz; $\Delta C(D_2) = 0.456$ ppm) [26] were also clearly evident in the ^{13}C NMR spectrum. The presence of non-deuterated molecules besides the dideuterated ones (ca. 28% as calculated from the integrated peak areas in the ^1H NMR spectrum, taking into account the number of protons in the two species) had to be attributed to additional acetate synthesis from CO_2 by the homoacetogenic bacterium according to the stoichiometry of processes reported Fig. 1a [15]. The outcome of this feeding experiment was indicative of the conversion of all the CH_2OH groups of TEA into the COO^- group of the resulting acetate as well as of the retention of both hydrogen atoms of the *N*-linked methylene groups, thus excluding the pathways b and c of Fig. 1.

When cells of strain LuTria3 were fed with 2,2′,2″-nitrilotri(1,1′,1″-$^2H_6$)ethanol (**14**) the resulting acetate was found to be a mixture of mono- and non-deuterated molecules (the latter being ca. 27%). In fact, in the ^1H NMR of this acetate an upfield 1:1:1 triplet ($^2J_{HD} = 2.01$ Hz; isotope shift, $^2\Delta H(D) = 13.0$ ppb) [22–24] was present besides the CH_3 singlet (Fig. 3b). An analogous pattern of signals was observed in the ^{13}C NMR spectrum ($J_{CD} = 19.5$ Hz; $\Delta C(D) = 0.232$ ppm) [25,26]. Identical spectra were obtained for acetate coming from feeding experiments using DEA and EA dideuterated at the hydroxymethyl group, i.e., **16** and **18**, respectively (see Fig. 2b for their preparation). The result of these experiments were consistent with the radical mechanism schematized in Fig. 1d, which has been proven to occur in a number of enzymatic reactions, all consisting of a formal interchange of a H-atom and a variable group between adjacent C-atoms. If such rearrangement gives rise to an acetal, a hemiacetal or a hemiaminal (such as **4a** and **b**), thereby to an aldehyde as overall product, the generic term eliminase has been proposed for the corresponding enzyme [27].

Considering that during the enzymatic rearrangement a partial solvent exchange of the mobile H-atom could occur (masked by the acetogenic activity of the bacterium in the second experiment), fully deuterated TEA (**11**) was synthesized (Fig. 2a) and fermented under the usual anaerobic conditions. The resulting sodium acetate was then examined by ^2H NMR and mass spectroscopy. The absence of significant amounts of partially deuterated molecules in such sample, as revealed by the very narrow singlet at 1.82 ppm due to $CD_3CO_2^-\ Na^+$ in the ^2H NMR spectrum (Fig. 3c), was confirmed by a FAB-MS analysis showing only two detectable peaks at *m/z* 105 $[CH_3COONa + Na]^+$ and *m/z* 108 $[CD_3COONa + Na]^+$.

The entire retention of the 1,2-migrating deuterium atom is typical of radical mediated enzymatic reactions (Figs. 1c,d). However in two cases of eliminase-catalyzed radical reactions a complete exchange of the mobile H-atom has been observed [28,29]. The biodegradation of the ethanolamines described here appears to be similar to the conversion of 2-phenoxyethanol into acetate mediated by another strain of the anaerobic homoacetogenic bacterium *Acetobacterium* [30,31]. While adenosylcobalamin (coen-

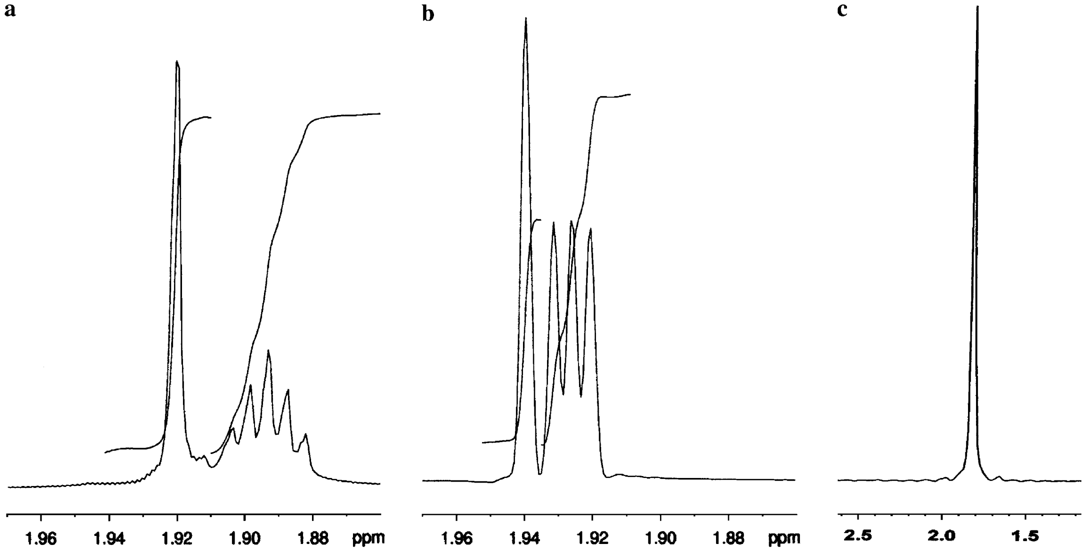

Fig. 3. Spectra of sodium acetate (methyl resonances only) produced by fermentation of deuterated triethanolamines **10** (a, ^1H NMR, 400 MHz), **14** (b, ^1H NMR, 400 MHz) and **11** (c, ^2H NMR, 61.4 MHz).

zyme B_{12}) and *S*-adenosylcobalamin (SAM) are well recognized as cofactors initiating most eliminase reactions [29], the radical initiators required by the *Acetobacterium* strains remain to be identified.

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