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SYNTHESIS OF TRIMETHYLATED PHOSPHONIUM AND ARSONIUM ANALOGUES OF THE OSMOPROTECTANT GLYCINE BETAINE; CONTRASTED BIOLOGICAL ACTIVITIES IN TWO BACTERIAL SPECIES

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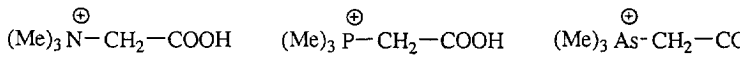
Abstract: Phosphoniobetaine and arsenobetaine, the P and As analogues of glycine betaine (trimethylammonioacetate) were synthesized and assayed for activity in bacterial osmoprotection biotests, using *Escherichia coli* and *Rhizobium meliloti* as model organisms. The P- and As-betaines displayed similar osmoprotective activities in *E. coli*, but were highly toxic in the betaine-demethylating bacterium *R. meliloti*.

To survive and grow in environments of low water activities, i.e., to cope with osmotic and salt stresses, bacteria must maintain a positive turgor to avoid cellular dehydration. A common strategy of osmoadaptation consists in the accumulation of submolar concentrations of small organic osmolytes, exclusively in stressed cells. Exogenously supplied osmolytes are termed osmoprotectants because they strongly stimulate the growth of stressed bacteria and are highly compatible with cellular functions at high cytosolic concentrations[1]. Glycine betaine **1** (GB, trimethylammonioacetate) is a powerful osmoprotectant because most bacteria possess high affinity betaine transporters. Thus, they can take advantage of micromolar levels of GB to grow in stressful environments[2]. Bacteria which use GB in osmoregulation belong to two classes. The enteric bacterium *Escherichia coli* is the representative model of the first class of bacteria which never metabolize GB[3]. The soil bacterium *Rhizobium meliloti* is the most studied example of the second class of bacteria that can metabolize GB very actively when grown at low osmolarities, but accumulate it at high osmolarities[4,5].

Here, we report on the synthesis of two analogues of the osmoprotectant GB, the new phosphoniobetaine **2** and arsenobetaine **3**, in which the trimethylammonium moiety Me_3N^+ of GB is replaced by a trimethylphosphonium group Me_3P^+, and a trimethylarsonium group Me_3As^+ (fig. 1), for **2** and **3** respectively. Also, we describe their contrasting biological activities in two bacterial models, *E. coli* and *R. meliloti*, which differ in their ability to catabolize GB. Arsenobetaine and phosphoniobetaine are highly beneficial (i.e., osmoprotective) in *E. coli*, but are extremely toxic in *R. meliloti*. Our data provide the first experimental evidence validating the concept of antibacterial betaines[6].



1 **2** **3**

Figure 1: Chemical structure of the three betaines used in this study: **1**, glycine betaine (GB); **2**, phosphonic-betaine (PB); **3**, arsenobetaine (AsB).

Phosphoniobetaine **2** was synthesized in 3 steps starting from reagent-grade triphenylphosphite **4** (Fig. 2). CAUTION: Preparation of trimethylphosphine **5** is the critical step in this sequence. Schlenk lines were necessary to synthesize this compound which is highly toxic and burns spontaneously in the presence of

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traces of O_2. Procedures based on addition of Grignard's reagents on PBr_3 give poor yields[7]. Therefore, trimethylphosphine **5** was prepared by slowly adding triphenylphosphite **4** to 3 equivalents of methylmagnesium iodide (MeMgI) in dibutyl ether. According to this procedure, decomposition of trimethylphosphine was avoided and a 65% yield was obtained. Addition of ethyl bromoacetate to a solution of trimethylphosphine **5** in ether led to the formation of a precipitate of phosphoniobetaine ethyl ester bromide **6**, which was purified by recristallization in acetone. The ester **6** was hydrolysed to phosphoniobetaine **2** by passage through a strongly basic anion exchange column (Dowex 1 x 8 / OH^-).

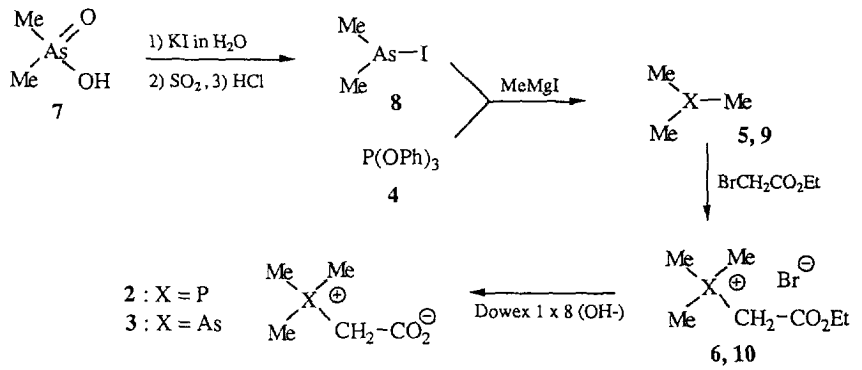

Figure 2: Synthesis of phosphoniobetaine (**2**) and arsenobetaine (**3**). For **2**, **5**, **6**, X = P. For **3**, **9** and **10**, X = As.

Arsenobetaine **3** was prepared in 4 steps according to a slightly modified literature procedure[8] (Fig. 2). The first step is the reduction of reagent grade cacodylic acid **7** with KI in water saturated with sulphur dioxide. Iodoarsine **8** thus obtained is methylated with MeMgI in ether at low temperature (-50°C). CAUTION: Trimethylarsine **9**, which is highly toxic was purified by trap-to-trap distillation using the apparatus[9] described in the figure 3. Addition of ethyl bromoacetate to a solution of trimethylarsine **9** in benzene led to the formation of arsenobetaine ethyl ester **10** which precipitates. Arsenobetaine **3** was obtained by hydrolysis of **10** as described above for **2**. The chemical identities of all synthetic compounds were ascertained by ^1H and ^{13}C, and ^{31}P NMR for **2** [10].

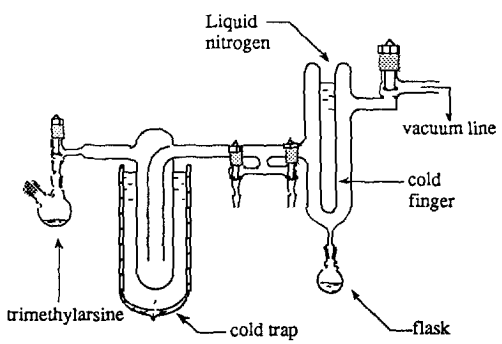

Figure 3: Scheme of the apparatus used for the purification of trimethylarsine **9**.

The growth of *E. coli* MC4100 is strongly inhibited by the addition of 0.8 M NaCl to the culture medium. However, growth can be restored (osmoprotection) by the uptake and intracellular accumulation of exogenously supplied osmolytes such as glycine betaine[1]. Fig. 4A shows that arsenobetaine and phosphoniobetaine, like GB, acted as powerful osmoprotectants in *E. coli* MC4100 grown with NaCl, but the three betaines had no effects on cells grown without NaCl. Moreover, chromatographic and electrophoretic analysis of ethanolic extracts[4] from *E. coli* grown with PB, AsB or GB, showed that the three betaines were accumulated as non-metabolizable osmolytes, exclusively in salt-stressed cells. Furthermore, competition for

GB uptake, and the use of mutants of *E. coli* that lack the transporters ProP, ProU or both[11] demonstrated that the uptake of PB in *E. coli* occurs *via* the general osmoporters ProP and ProU which are involved in the uptake of GB, AsB and all other known osmoprotectants[6, 11] (data not shown). In all, our data clearly demonstrate that PB, AsB and GB are mutually interchangeable as cytosolic osmolytes which alleviate osmotic inhibition of growth in *E. coli*.

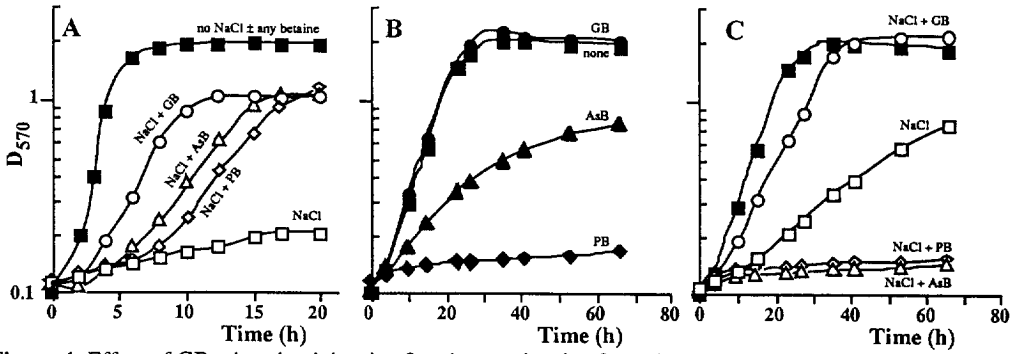

Figure 4: Effect of GB, phosphoniobetaine **2** and arsenobetaine **3**, on the growth of *E. coli* MC4100 (A) and *R. meliloti* 102F34 (B and C) at low and high osmolarities. Panel A. *E. coli* MC4100 was grown aerobically at 37°C in minimal M63 medium[11] with or without 1 mM of one of the three betaines (■). Osmotic stress was imposed by adding 0.8 M NaCl (open symbols) to cultures grown without added betaine (□), or with 1 mM of **1** (○), **3** (△) or **2** (◇). D_{570}, bacterial growth expressed as the attenuance at 570 nm (log scale). Panels B and C. *R. meliloti* 102F34 was grown aerobically at 30°C in minimal LAS medium[4] with (open symbols) or without (closed symbols) 0.5 M NaCl. □, no betaine added; ○, 1 mM of **1** added; △, 1 mM of **3** added; ◇, 1 mM of **2** added.

Arsenobetaine and phosphoniobetaine were also tested in osmoprotection bioassays in *R. meliloti* wild-type strain 102F34. Again, GB was used as a positive control in the growth tests[4]. Fig. 4B shows that the three betaines displayed contrasting biological activities in control cultures grown without NaCl. Indeed, GB was inert, but AsB and PB were highly toxic. Strikingly, 1 mM AsB was about as inhibitory as a high osmotic stress imposed by 0.5 M NaCl, and PB prevented growth of *R. meliloti* 102F34 in low-osmolarity medium (Figs. 4B, 4C). These data contrasted sharply with those obtained with unstressed cultures of *E. coli* MC4100 in which the three betaines were physiologically inert (Fig. 4A). As expected[4], GB was highly osmoprotective in *R. meliloti* 102F34, restoring its growth rate and cell yield almost to the unstressed levels. Moreover, the toxic effects of AsB and NaCl were additive. Indeed, no growth was observed when *R. meliloti* 102F34 was inoculated in 0.5 M NaCl plus AsB. Similarly, no growth occurred when PB was substituted for AsB in the high-osmolarity medium (Fig. 4C). Also, it is noteworthy that the ethyl esters **6** and **10** and the corresponding betaines **2** and **3** displayed similar biological activities, i.e., osmoprotection in *E. coli* MC4100 and toxicity in *R. meliloti* 102F34, respectively. Moreover, both strains converted the esters **6** and **10** to **2** and **3**, respectively, (data not shown), suggesting the action of a betaine esterase in the two bacterial species.

R. meliloti 102F34, unlike *E. coli* MC4100, can metabolize GB and assimilate it as a source of carbon and nitrogen[4]. Thus, the biological activity of the three betaines was also assayed in a spontaneous mutant of *R. meliloti*, VP01, that does not use GB as a growth substrate. Interestingly, phosphoniobetaine and arsenobetaine had no effects on the unstressed mutant and could substitute for GB as powerful osmoprotectants and cytosolic osmolytes in salt-stressed cells (data not shown). In all, our data strongly suggest that the very high toxicity of PB and AsB in *R. meliloti* wild-type strain 102F34 is indirect and stems from their catabolism *via* the glycine betaine demethylation pathway which was characterized in this strain[5].

In summary, we report on the synthesis of arsenobetaine **3** and the new betaine, phosphoniobetaine **2**. Also, we show that the two structural analogues of the universal osmoprotectant GB[1,3] **1**, display contrasting biological activities in two bacterial species which differ in their ability to metabolize GB: phosphoniobetaine and arsenobetaine are highly toxic to wild-type *R. meliloti* 102F34, but are highly beneficial (e.g. osmoprotective) to salt-stressed cultures of wild-type *E. coli* MC4100, and in a mutant of *R. meliloti* which are both unable to catabolize GB. Clearly, these data demonstrate that PB and AsB, like GB, can behave as genuine compatible solutes and osmoprotectants, at least in bacteria that can not demethylate these betaines.

We are currently investigating the possibility of a broader toxicity of arsenobetaine and phosphoniobetaine in bacteria; particularly in species and strains that metabolize GB via serial demethylations or other catabolic pathways. To the best of our knowledge, this is the first report on the antibacterial activity of any betaine. We believe that our data may open new directions in betaine research. Indeed, it was recently proposed that the avid affinity of betaine porters for their substrates might be used to deliver antibacterial betaines intracellularly to treat bacterial infections. This study provides the first experimental evidence validating this proposal and the concept of antibacterial betaines. The characterization of the genes and enzymes involved in the catabolism of GB in *R. meliloti* 102F34 should facilitate the identification of the toxic metabolites of **2** and **3**, which might also be toxic in other bacterial species. Lastly, this research opens the way to the design of new betaines or betaine derivatives with wider antibacterial specificities and potential therapeutic applications[6,12], and to the comprehension of their mode of action in bacteria.

Acknowledgements

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10. **2** : ^{31}P NMR (D_2O) d: 23.9 (s). ^{1}H NMR (D_2O): d 1.95 (d, $^{2}J_{PH}$ = 14.6 Hz, ($C\underline{H}_3)_3P$), 3.2 (d, $^{2}J_{PH}$ = 14.4 Hz, $C\underline{H}_2P$). ^{13}C NMR (D_2O): d 10.4 (d, $^{1}J_{CP}$ = 56.5 Hz, ($\underline{C}H_3)_3P$), 33.2 (d, $^{1}J_{CP}$ = 58.6 Hz, $\underline{C}H_2P$), 172.2 (C=O). **3** : ^{1}H NMR (D_2O): d 1.9 (s, ($C\underline{H}_3)_3As$), 3.35 (s, $C\underline{H}_2As$). ^{13}C (D_2O): d 8.1 (($\underline{C}H_3)_3As$), 34.1 ($\underline{C}H_2As$), 173.1 (C=O).
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