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TOXICITY AND OSMOPROTECTIVE ACTIVITIES OF ANALOGUES OF GLYCINE BETAINE OBTAINED BY SOLID PHASE ORGANIC SYNTHESIS TOWARDS *SINORHIZOBIUM MELILOTI*

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Abstract: Seven analogues of the bacterial osmoprotectant glycine betaine (GB, trimethylammonioacetate), in which the methyl groups of the Me_3N^+ moiety are replaced by various substituents, were obtained by SPOS using Wang resin. Their biological activities (osmoprotection vs toxicity), appeared closely related to their uptake efficiency and their catabolism in the betaine-demethylating model bacterium *Sinorhizobium meliloti*.

Glycine betaine **1** (GB, trimethylammonioacetate) is a common osmolyte synthesized by marine algae[1] and halophilic plants.[2] GB is also a universal bacterial osmoprotectant that stimulates growth and proliferation of numerous species of bacteria in hyperosmotic environments such as seawater and media containing inhibitory concentrations of salts or organic compounds. GB alleviates osmotic stress because it accumulates to high cytosolic concentrations and, therefore, counteracts cell dehydration by restoring a net positive cell turgor pressure (cytoplasm > medium) which drives bacterial growth.[3] Like GB, dimethylsulfonioacetate **2** (DMSA), arsenobetaine (AsB) and phosphoniobetaine (PB) (*i.e.*, the S-, As- and P-analogues of GB, respectively) are highly osmoprotective for several bacteria including the model species *Escherichia coli*.[4,5] GB also enhances salinity tolerance in the model soil bacterium *Sinorhizobium meliloti*.[6] In sharp contrast, DMSA, AsB and PB are not osmoprotective for this bacterium. Indeed, these three compounds are highly toxic and strongly inhibit the growth of *S. meliloti* wild-type strain 102F34.[5,7] These observations are extremely interesting because no antibacterial activity had been described before for any natural or synthetic betaine in any bacterium, although AsB might be toxic to yet unidentified marine bacteria.[8] Furthermore, it was proposed recently that new synthetic betaines with antibacterial activities could possibly be designed and used to treat bacterial infections.[9] Therefore, we sought to modify the chemical structure of GB **1** by replacing its N-linked methyl groups by different sets of substituents. In this communication, we describe an improved and simplified solid phase organic synthesis (SPOS) protocol that allows the rapid synthesis and the obtention of maximal yields of new derivatives of GB. Also, we compare the biological activities of seven of these synthetic betaines to those of GB and DMSA in two model bacteria (*S. meliloti* and *E. coli*) with well characterized physiological responses to GB and DMSA, a rare algal sulfonium analogue of GB.[7]

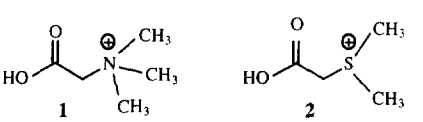

Figure 1. Chemical structures of glycine betaine **1** (GB) and dimethylsulfonioacetate **2** (DMSA).

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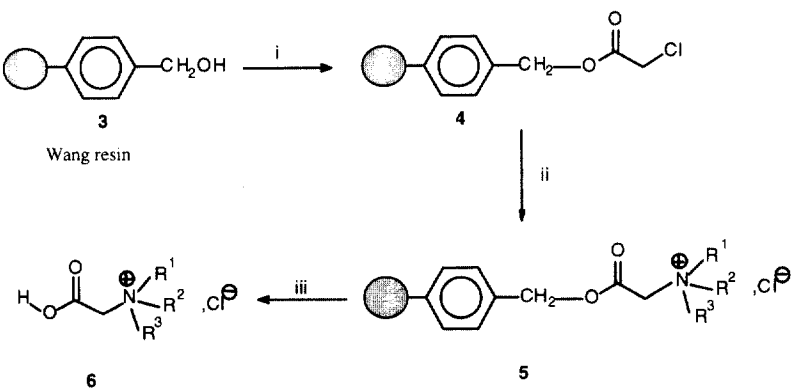
1. Obtention of modified glycine betaines by solid phase organic synthesis

Published procedures for liquid phase synthesis of GB and similar compounds involve the peralkylation of amino acids[10] or the addition of an ammonium salt[11] or a dialkyl sulfide[12] to either acrylic acid or bromoacetic acid.[13] Recently, SPOS has emerged as a powerful tool that allows for a simplification of reaction procedures and the suppression of many purification steps which are required in solution phase chemical synthesis.[14] Here, the liquid phase methodology described by Lloyd *et al.*[15] was adapted and improved in order to set up a standard SPOS protocol that would allow for the rapid synthesis of a large number of structural analogues of GB. Briefly,[16] a Wang resin **3** was esterified with chloracetyl chloride in the presence of pyridine,[17] yielding the halogenated polymer **4** (Fig. 2). Then, 1-g fractions of this polymer were subjected to reaction with 5 molar equivalents of the appropriate tertiary amine, leading to the anchored ammonium salt **5**. The treatment of the ester **5** by TFA released the trifluoroacetate salt of the synthetic betaine which was solubilised in 2 M aqueous HCl and recovered as a betaine hydrochloride (**6**) after evaporation of HCl and trituration in dimethylketone (Fig. 2).



i: chloracetyl chloride, pyridine ii: tertiary amine,DMSO iii: 1)TFA 2) aqueous HCl

Figure 2. Chemical route for solid phase synthesis of structural analogues of GB (detailed reaction conditions are given in footnote n° 16).

The limiting step in the above reaction sequence is the addition of the tertiary amine to the halogenated polymer **4**. Ideally, the SPOS solvent must induce a good swelling of the resin and also favour the nucleophilic substitution of the halogen by the tertiary amine. Various organic solvents were tested in the presence of isopropyldimethylamine as the incoming amine. Dichloromethane afforded a reaction yield of only 18% (Table 1) although this solvent induced a satisfactory swelling of the polymer. Moderate yields were obtained when the addition reaction was repeated a second time in the presence of fresh CH_2Cl_2 and isopropyldimethylamine, or when the reaction was carried out either in DMF or in CH_2Cl_2 supplemented with a small amount of either DMSO or CH_3CN. A higher addition yield was obtained in pure CH_3CN and a maximal yield was observed, as expected, in pure DMSO, which was used as the solvent of the addition reaction in the syntheses described below.

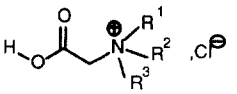
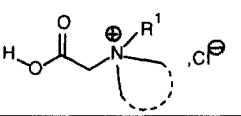
Table 1. Solvent effect on the addition reaction of isopropyldimethylamine to the halogenated polymer **4**.[(a)]

Solvent[(b)]	CH_2Cl_2	DMF	CH_2Cl_2:DMSO[(c)]	CH_2Cl_2:CH_3CN[(c)]	CH_3CN	DMSO
Yield (%)	18; 45[(d)]	40[(e)]	45	40	70	95

[(a)] Isopropyldimethylamine [$(CH_3)_2N$-$CH_2(CH_3)_2$] was used as the model of tertiary amine. [(b)] DMF, dimethylformamide; CH_2Cl_2, dichloromethane; DMSO, dimethylsulfoxide; CH_3CN, acetonitrile. [(c)] The proportions of the two solvents were 9:1 (vol/vol). [(d)] The reaction was carried out twice with a 5-molar equivalent of isopropyldimethylamine. [(e)] A side reaction led to the formation of *N,N*-dimethylglycine hydrochloride (yield 30%), most probably due to the presence of dimethylamine in DMF.[18]

Seven analogues of GB were obtained by solid-phase synthesis in DMSO. These betaines (**61** to **67**) differed from GB **1** by the substitution of one to three of its methyl groups by various substituents (Table 2).

Table 2. Chemical structures of the synthetic GB analogues obtained by SPOS.

General formula:  

	61	**62**	**63**	**64**	**65**	**66**	**67**
R^1	$-CH_3$	$-CH_3$	$-CH_2CH_3$	$-CH_3$	$-CH_3$	$-CH_3$	$-CH_3$
R^2	$-CH_3$	$-CH_2CH_3$	$-CH_2CH_3$	$-CH_3$	$-CH_3$		
R^3	$-CH_2CH_3$	$-CH_2CH_3$	$-CH_2CH_3$	$-CH(CH_3)_2$	$-(CH_2)_3CH_3$		

The chemical names of the synthetic betaines are given in footnote n° 16. The chemical structures of these compounds were ascertained by ^1H NMR, ^{13}C NMR and IR spectroscopies, and their purity was evaluated by high-voltage paper electrophoresis as well as chromatography techniques.[19]

2. Comparative biological activities of GB, DMSA and seven synthetic GB analogues

Bacterial growth in liquid minimal media was monitored spectrophotometrically to evaluate the biological activities of the synthetic analogues of GB in strains of *S. meliloti* and *E. coli* that differ from each other by their abilities to transport and/or catabolize GB, DMSA, AsB and PB.[5] Cultures were grown aerobically either in LAS medium[6] at 30°C (*S. meliloti*) or in M63 medium[20] at 37°C (*E. coli*) in the presence of 1 mM GB, DMSA or one of the seven synthetic betaines (**61** to **67**). Osmotic stress was applied by adding NaCl into the growth media (0.5 and 0.8 M NaCl for *S. meliloti* and *E. coli*, respectively).

Table 3. Comparative effects GB, DMSA and synthetic analogues of GB on the growth of *S. meliloti* 102F34 at low and high osmolarities

Betaine added at 1 mM	**Growth parameters in LAS medium**			
	without NaCl		**with 0.5 M NaCl**	
	DT (h)	*Dmax*	DT (h)	*Dmax*
None	5.0	1.9	20	0.9
GB 1	5.5	2.0	7.0	2.1
DMSA 2	35	0.5	32	0.5
61	35	0.7	11	2.0
62	9.0	1.6	13	2.0
63	7.9	1.8	18	0.9
64	6.9	1.8	20	0.9
65	6.4	1.9	20	0.9
66	45	0.6	11	2.1
67	28	1.0	13	1.9

Cultures were grown in lactate-aspartate-salts (LAS) minimal medium.[6] Bacterial growth was monitored spectrophotometrically by measuring the attenuance (D) of cell suspensions at 570 nm. DT is the doubling time of the culture (in hours per generation) in the mid-exponential phase of growth. *Dmax* is the maximal attenuance reached in the stationary phase. Each value is the mean from duplicate determinations, with less than 10 % error.

The growth rate and yield of *S. meliloti* 102F34 decreased dramatically in high-osmolarity LAS medium containing 0.5 M NaCl. As expected,[6,7] GB was physiologically inert in the unstressed culture grown in LAS without NaCl, but relieved growth inhibition due to high salinity (Table 3). In contrast to GB, DMSA **2** was highly toxic to cells cultured in LAS without NaCl, and did not confer enhanced salinity tolerance to *S. meliloti* 102F34. Interestingly, **61**, **66**, and **67** were also highly toxic to cultures grown in low-osmolarity LAS medium, reducing their growth rates 7, 9 and 5.6 fold, respectively (Table 3). However, unlike DMSA, the synthetic betaines **61**, **66**, and **67** were osmoprotective and restored the final growth yield of salt-stressed cultures to the level observed in the culture grown without NaCl, although **61**, **66** and **67** displayed a less pronounced stimulatory effect than GB on the growth rate of salt-stressed cells. Meanwhile, the diethylated (**62**), the triethylated (**63**) as well as the monoisopropyl (**64**) and monobutyl (**65**) glycine betaines displayed only a weak inhibitory effect on unstressed cultures of *S. meliloti* 102F34. Furthermore, of these four compounds only the diethylated betaine (**62**) conferred enhanced salinity tolerance to this strain, while **63**, **64** and **65** were physiologically inert in cultures grown at high salinity (Table 3). Also, as described previously for DMSA, AsB and PB[5,7] **61**, **66**, and **67** were not toxic for unstressed cultures of *S. meliloti* mutant strain VP01 [derived from strain 102F34] which is impaired in its ability to catabolize GB, DMSA, AsB and PB.[7] In summary, the data presented above demonstrate that three synthetic derivatives of GB **1** (**61**, **66** and **67**), like AsB and PB,[5] were highly toxic and inhibited the growth of *S. meliloti* wild-type strain 102F34 at low osmolarity. However, **61**, **66** and **67** [in contrast to AsB and PB which were also inhibitory for salt-stressed cultures of strain 102F34][5] were osmoprotective, *i.e.*, enhanced the salinity tolerance of this strain.

The biological activities of the seven synthetic betaines (**61** to **67**) were also evaluated in a wild-type strain of *E. coli* (MC4100) which is unable to catabolise GB and its S-, As- and P-analogues.[5] As observed previously with AsB and PB,[5] DMSA and the seven synthetic GB analogues (**61** to **67**) were physiologically inert (*i.e.*, displayed no toxicity) towards unstressed cultures of MC4100 grown in M63 medium without NaCl. However, all of these compounds were highly osmoprotective for stressed cells of MC4100 grown in M63 medium containing 0.8 M NaCl. Lastly, like GB and many other bacterial osmoprotectants (AsB, PB, etc.),[5,20] DMSA and the seven synthetic GB analogues (**61** to **67**) were not osmoprotective for *E. coli* MKH13[21] (data not shown). Interestingly, MKH13 is a mutant derivative of *E. coli* MC4100 which is unable to accumulate exogenous GB and DMSA because the two transporters (ProP and ProU) involved in the uptake of GB and many other osmoprotectants in MC4100 were deleted in strain MKH13.[21] Thus, these data collectively demonstrate that the osmoprotective activities of the seven synthetic betaines (**61** to **67**) in *E. coli* were linked to the presence of functional betaine transporters.

Table 4. Substrate specificity of betaine uptake in salt-stressed *S. meliloti*.

Competitor added	Competitor/substrate ratio		
	1:1	10:1	100:1
1	80	94	99
2	52	90	98
61	35	79	96
62	4	10	57
63	0	4	27
64	0	4	45
65	1	10	23
66	20	61	95
67	1	7	43

Uptake experiments were performed as described.[7] Data are expressed as percentages of inhibition of [^{14}C]DMSA uptake, compared with the uninhibited transport rate which was 30 nmol/min/mg of protein.

Competition studies were performed to evaluate the ability of GB and synthetic betaines to inhibit the uptake of radioactive DMSA by salt-stressed *S. meliloti* 102F34. [*methyl*-^{14}C]DMSA was used at a

saturating concentration of 50 μM and each of the putative competitors was supplied at competitor/substrate ratios of 1:1, 10:1 and 100:1. Table 4 shows that GB inhibited [^{14}C]DMSA uptake more efficiently than unlabelled DMSA inhibited its own uptake (80 and 52% inhibition by an equimolar concentration of GB and DMSA, respectively). The monoethylated (**61**) and pyrrolidinyl (**66**) betaines also inhibited DMSA uptake significantly when [^{14}C]DMSA and either **61** or **66** were used at a concentration of 50 μM (35 and 20% inhibition by **61** and **66**, respectively). Moreover, a 100-fold excess of either **61** or **66** virtually prevented the uptake of [^{14}C]DMSA. Meanwhile, a 10-fold molar excess of either **62**, **63**, **64**, **65** or **67** had little effect on DMSA uptake, but these five synthetic betaines were significantly inhibitory (23 to 57% inhibition) when used at a 100-fold molar excess (Table 4). In all, the results of these competition studies indicate that: (i) the seven synthetic GB analogues were also taken up via GB/DMSA transporters in *S. meliloti*, and (ii) the compounds which had little effect on sinorhizobial growth (**62**, **63**, **64** and **65**) were not transported efficiently by these porters, except when used at rather high (millimolar) concentrations.

Several lines of evidence in this study demonstrate that the biological activities (toxicity and/or osmoprotection) of the seven synthetic analogues of GB (**61** to **67**) are closely related to their uptake via GB/DMSA porters: (i) all of these compounds are physiologically inert in a mutant strain of *E. coli* (MKH13) that lacks functional GB porters, but are highly osmoprotective for the parental strain (MC4100), (ii) **61** and **66** which strongly inhibit the growth of *S. meliloti* 102F34 at low osmolarity (Table 3) are by far the strongest inhibitors of DMSA uptake in this strain (Table 4), and (iii) synthetic betaines that have little effect on sinorhizobial growth (**62**, **63**, **64** and **65**) do not inhibit DMSA uptake significantly, except when used at a 100:1 molar excess (Tables 3,4).

Furthermore, as observed with DMSA, AsB and PB,[5,7] the toxicity of **61**, **66** and **67** for *S. meliloti* 102F34 (grown at low osmolarity) stems from their catabolism via the GB/DMSA demethylation pathway which is absent in *E. coli*.[3,6,22] In *S. meliloti*, this catabolic pathway is constitutively expressed in a low-osmolarity medium, and is substrate inducible.[6,22] Osmotic stress dramatically reduces enzymatic activities in this pathway, and betaine accumulation in salt-stressed *S. meliloti* results from the predominance of osmotic inhibition of this pathway over substrate inducibility.[22,23] Considering these physiological features, the surprising behaviour of **61**, **66** and **67** (toxic at low osmolarity, and osmoprotectant at high osmolarity) should correspond to that of a good substrate, which would be unable to induce the catabolic pathway.

3. Conclusion

The data presented in this communication validate the proposal of using GB as a core structural motif to design new antibacterial drugs.[9] Thus, our data should be of clinical interest because they suggest that the ability of pathogenic bacteria to catabolize potentially toxic GB analogues will determine the intensity of the antibacterial activities and the spectrum of the therapeutic potentialities of these compounds. In other words, our data bring a first contribution to the definition of the structural parameters that will be important for the development of new antimicrobial betaines with higher and broader antibacterial activities than **61**, **66** and **67**. Prominent among these parameters will be the affinities of betaine porters for these compounds. With this end in view, the SPOS method developed in this work, which can be easily automatisable, will be convenient to prepare large libraries of betaine analogues (in which the diversity would come from the nature of the tertiary amine), with elevated yields and high purity.

Acknowledgments

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16. **Synthesis of polymer 4:** Two grams of Wang resin **3** (1.24 mmol), were mixed with 2 molar equivalents of pyridine and chloracetyl chloride in 45 ml of dry CH_2Cl_2, at 0°C under a nitrogen flow. The esterification reaction was carried out by stirring the suspension overnight at room temperature. Then, the esterified polymer **4** was washed 5 times in 10 ml of CH_2Cl_2 and 3 times in each of the following solvents: 10 ml of tetrahydrofuran (THF), 20 ml of THF/water (1/1, vol/vol), 20 ml of water, 20 ml of THF, 10 ml of CH_2Cl_2, and twice in 10 ml of diethylether which was evaporated under vacuum. **Synthesis of polymer 5:** The purified polymer **4** (1g; 0.62 mmol) was resuspended in 7 ml of dry DMSO under a nitrogen flow. Then, a solution of the appropriate tertiary amine (5 molar equivalents in 2 ml of DMSO) was added and the suspension was stirred 18 h and washed as described above. **Obtention of the synthetic betaine 6:** The washed polymer **5** was subjected to cleavage by 6 ml of TFA in CH_2Cl_2 (1/1, vol/vol), yielding the trifluoroacetate salt of the synthetic betaine. Then, the resin was filtered and washed 5 times with 5 ml of CH_2Cl_2. The combined organic filtrates were evaporated under vacuum and the dried residue was triturated in water. The aqueous suspension was washed twice with 3 ml of CH_2Cl_2. Then, water was evaporated and the chlorhydrate salt of the synthetic betaine was obtained by stirring the dry residue for 2 h in 5 ml of 2 M aqueous HCl. Finally, the synthetic betaine hydrochloride was purified further by triturating the white solid in dimethylketone. Seven GB analogues [**N°**: name (yield%)] were synthesised in this study: **61**: carboxymethyldimethylethyl ammonium chloride (100%), **62**: carboxymethyldiethylmethyl ammonium chloride (95%), **63**: carboxymethyltriethyl ammonium chloride (65%), **64**: carboxymethyldimethylisopropyl ammonium chloride (95%), **65**: carboxymethylbutyldimethyl ammonium chloride (80%), **66**: carboxymethylmethylpyrrolidinyl ammonium chloride (75%) and **67**: carboxymethylmethylmorpholinyl ammonium chloride (65%).
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