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Antibacterial activity of glycine betaine analogues: involvement of osmoporters

Anne Cosquer,[a] Morgane Ficamos,[b] Mohamed Jebbar,[a] Jean-Charles Corbel,[b] Gwénaëlle Choquet,[a] Catherine Fontenelle,[a] Philippe Uriac[b] and Théophile Bernard[a,*]

[a]*Université de Rennes I, UMR CNRS 6026, Interactions Cellulaires et Moléculaires, Equipe Osmoadaptation chez les Bactéries, Campus de Beaulieu, 35042 Rennes, France*
[b]*Institut de Chimie de Rennes, Synthèse et Extraction de Molécules à visée Thérapeutique, UPRES 2234, Faculté de Pharmacie, 2 Avenue du Pr L. Bernard, 35043 Rennes, France*

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Abstract—Glycine betaine (GB) analogues were obtained using solid phase organic synthesis and assayed for their toxic activity against 15 Gram positive and Gram negative bacteria. Four benzyl derivatives of GB were selected to determine their effect on bacterial growth. Bacteriostatic and lethal effects were observed for compound **1** and compound **2**, respectively. The importation of the two GB analogues into bacterial cells appeared strictly dependent on the presence of the powerful betaine membrane osmoporters; their capacity to be amassed intracellularly at molar levels from extremely dilute solutions might constitute a basis to design a new class of antimicrobial agents.
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1. Introduction

Numerous species of bacteria cope with elevated osmolality by importing compatible solutes (osmoprotectants) from their environment.[1–3] Glycine betaine (*N*,*N*,*N*-trimethylglycine, GB), the most powerful osmoprotectant identified so far, is internalised through unspecific but highly efficient membrane carriers (osmoporters) and can be amassed intracellularly up to molar levels[2] (i.e., 10^3–10^9 times that of the medium) depending on the degree of the osmotic constraint and the concentration of the osmoprotectant in the medium.[4] Examples of the most studied osmoporters are provided by ProU and ProP in the Gram negative models *Escherichia coli* and *Salmonella typhimurium*[1,5] and by OpuA, OpuC and OpuD in the Gram positive model *Bacillus subtilis*.[6] All these membrane carriers display a high affinity towards glycine betaine allowing concentration as low as one nanomolar to be effective in osmoprotection;[4] moreover these carriers are strikingly activated when bacterial cells have to face hyperosmotic conditions.

Such properties suggest that betaine porters could be very efficient ways to deliver toxic GB analogues, at a lethal level, into pathogenic bacteria, particularly those invading an hyperosmotic environment like the urinary tract,[7] salted foods, plant leaves and animal skin surfaces. Interestingly it has already been reported that structural analogues of GB exert a toxic activity against the Gram negative soil bacterium and plant microsymbiont *Sinorhizobium meliloti*.[8]

In this paper we report upon seven novel GB analogues obtained by solid phase synthesis and bearing a substituted benzyl moiety in the place of one methyl group. Two of these GB analogues were recently shown to express a cytotoxic activity against pathogenic strains of *E. coli*.[9] Thus, in order to perform a more complete biological study, we selected four compounds, which were prepared on a larger scale, using a classic method for synthesis, and assayed for their antimicrobial activities against 15 Gram negative and Gram positive bacterial species.

2. Chemistry

In our previous work dedicated to synthesis of GB analogues,[8] alkylation of Wang resin supported

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* Corresponding author. Tel.: +33-2-2323-6853; fax: +33-2-2323-6775; e-mail: tbernard@univ-rennes1.fr

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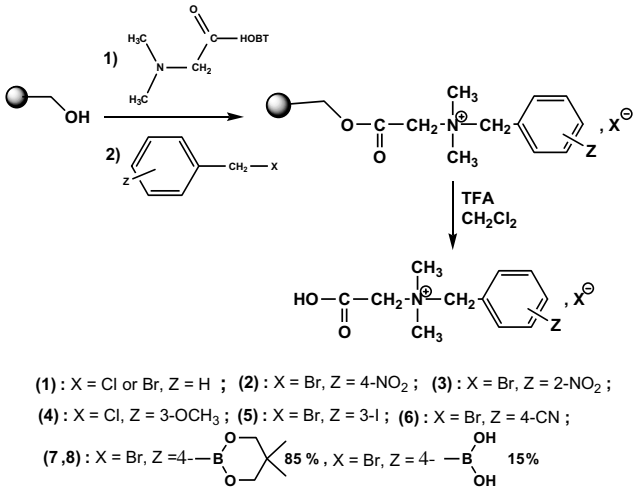
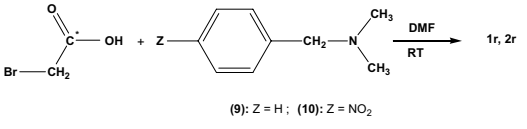


Scheme 1.

bromoacetic acid with tertiary amines was used. Here, another synthetic pathway was employed consisting of alkylation of dimethylglycine[10] linked to Wang resin by an ester (Scheme 1). Complete linkage of dimethylglycine to Wang resin (assessed using FTIR and ^{13}C gel NMR) was achieved after activation as HOBT ester, which also enabled dimethylglycine dissolution. Then, diversity was introduced with selected benzylhalides, which were either commercially available or prepared according to the literature.[11] During the cleavage, performed using a dichloromethane solution of trifluoroacetic acid, the bromide (or chloride) anion was not exchanged with trifluoroacetate anion, as demonstrated by MS (negative mode). Structure and purity[12] were checked by ^1H NMR, ^{13}C NMR, high-voltage electrophoresis on Whatman 3 MM paper[13] and chromatography (TLC and HPLC). This method[14] allowed us to prepare seven GB analogues (**1**–**7**) with very good yields (>90%) and with a high level of purity (>95%). The preparation of larger amounts of compounds **1**–**4** for the biological studies was performed by alkylation of the dimethylglycine ethyl ester according to the literature.[15]

In addition, ^{14}C radiolabelled compounds **1r** and **2r** were synthesised (Scheme 2) by direct alkylation of radiolabelled ^{14}C [*carboxy*]bromoacetic acid in DMF, using an excess of tertiary amines **9** and **10**. The amine **10** was prepared by alkylation of dimethylamine by 4-nitrobenzylbromide.[16] Purification of **1r** and **2r** was achieved using preparative high-voltage electrophoresis.[13]



Scheme 2.

3. Biological evaluation

A preliminary broad screening carried out to determine the effect of the seven synthesised GB analogues on bacterial growth (data not shown) led to the selection of the four most active compounds: **1**, **2**, **3** and **4**. In addition, a MIC distribution of **1** and **4** against 82 strains of *E. coli* isolated from infected urinary tracts has already been reported.[9]

Fifteen bacterial strains (listed in Table 1) were assayed for their growth in the presence of these compounds. The experiments were conducted aerobically in classic defined media (M63, M9, SMM or LAS), using NaCl to impose hyperosmotic conditions (0.3–0.5 M final concentration, depending on the degree of tolerance of the bacterium). Growth yield for each culture was expressed as the absorbance (optical density) of the suspension at 570 nm (A_{570}). GB and its analogues were supplied at 1 mM each.

On the basal medium, without added NaCl, except for *S. enterica* treated with compounds **3** and **4**, no significant growth inhibition was observed (data not shown). Under osmotically stressing conditions (Table 1), addition of compound **2** to the culture medium was followed by a severe loss of biomass production for a large majority (12 out of 15) of the species assayed; almost no growth (more than 92% inhibition) was observed for *A. hydrophila*, *C. freundii*, *E. chrysanthemi*, *E. coli*, *S. enterica* and *S. marcescens*. Only *B. linens*, *E. faecalis and S. meliloti* have their growth either not affected or rather slightly improved in the presence of **2**. The level of bacterial production, following treatment with **3,** was almost identical to that resulting from treatment with **2**. It should be mentioned that only *B. subtilis* was insensitive to that GB analogue.

Strains for which the growth was inhibited by **1** are also those affected by **2** except *B. subtilis*, *C. freundii*, *E. cloacae* and *S. enterica*. In contrast, compound **1** exerted a beneficial effect on the growth of *C. freundi* and *S. meliloti* with 21% and 57% yield improvement, respectively. Among the four analogues assayed, compound **4** showed the lowest toxicity. It appeared more or less effective (more than 27% growth inhibition) against *A. hydrophila*, *C. glutamicum*, *E. chrysanthemi*, *E. coli*, *K. pneumoniae* and *S. marcescens*.

Thus, for most of the strains submitted to treatment, compounds **1** and **2**, and to a lesser extent compound **3** caused the most inhibitory effect. Only *S. meliloti* and *E. faecalis* were not responsive to any of the GB analogues.

To determine whether an active transport, through betaine carriers, was required to cause bacterial toxicity, mutants were used. *E. coli* wild-type MC4100 (WT) and its derivatives BK 32 *(proP*), GM50 (*proU*) and MKH13 (*proU proP*) affected in osmoporters ProU and/or ProP synthesis were grown on the minimal medium M63 with glucose as the carbon and energy sources. Elevation of medium osmolality was made by addition of NaCl to a final concentration of 0.3 M. Betaine analogues were supplied at 1 mM each, as required.

Addition of salt to the culture medium altered the growth rate significantly in all the *E. coli* strains assayed

Table 1. Effect of structural analogues of glycine betaine (GB) on bacterial growth under stressing conditions[a]

Bacteria	Betaines				
	0	**1**	**2**	**3**	**4**
Acinetobacter baumannii	1.14	0.93	**0.19**	0.72	1.16
Aeromonas hydrophila	1.65	**0.16**	**0.06**	**0.20**	1.05
Bacillus subtilis	3.20	3.20	**0.18**	3.06	3.20
Brevibacterium linens	1.76	**0.88**	1.74	1.52	1.45
Citrobacter freundii	1.09	1.32	**0.03**	**0.06**	1.23
Corynebacterium glutamicum	0.87	**0.43**	**0.33**	**0.26**	**0.63**
Enterobacter cloacae	1.25	1.05	**0.24**	**0.46**	1.11
Enterococcus faecalis	1.16	1.24	1.23	1.30	1.12
Erwinia chrysanthemi	1.97	**0.22**	**0.06**	**0.11**	**0.25**
Escherichia coli	1.24	**0.12**	**0.09**	**0.08**	**0.18**
Klebsiella pneumoniae	1.80	**0.46**	**0.23**	**0.40**	**0.70**
Pseudomonas aeruginosa	1.24	**0.06**	**0.16**	**0.11**	1.07
Salmonella enterica	1.23	1.24	**0.07**	**0.23**	**1.11**
Serratia marcescens	1.03	**0.21**	**0.07**	**0.09**	**0.48**
Sinorhizobium meliloti	1.46	2.30	1.85	1.90	1.70

GB derivatives (**1**, **2**, **3** and **4**) were supplied at 1 mM each. Bacterial yield was expressed as absorbance (OD) at 570 nm.

[a] Defined classic culture media: M63 with glucose as carbon source except for *Enterococcus* (M9 medium), *S. meliloti*, *P. aeruginosa* and *B. linens* (LAS medium) and *B. subtilis* (SMM). NaCl concentration was adapted to each strain to allow a significant growth yield (0.5 M for *Bl*, *Ef* and *Pa*, 0.3 M for *Ec*, and 0.4 M for all other species). Bold numbers indicate the most prominent growth inhibiting activities. Data represent the average of at least three experiments; deviation was within 10%.

(Fig. 1). About a 50% decrease (from 0.85 to 0.46 generation/hour without and with added NaCl, respectively) was observed. Supplying **1** and **2** to their non-stressing medium (M63 0 M NaCl) did not affect the behaviour of either MC4100 or that of the three mutants (data not shown). An elevation of medium salinity (osmolality) in the presence of the two GB analogues led to a dramatic slow down of the growth rate of MC4100, BK32 and GM50; in fact only weak growth was observed in the presence of **1** and no growth in the presence of **2**. Interestingly, MKH13, the mutant deprived of both osmoporters appeared insensitive to **1** and **2** treatments. Identical conclusions were drawn from experiments carried out on *E. chrysanthemi* double mutant (*ousA*, *ous B*) and *B. subtilis* mutant (*opuC*); all these strains are known to be affected in betaine transport. Moreover, withdrawing compounds **1** and **2** from the salted medium permitted bacterial growth to resume for cells treated with **1**; in contrast those treated with compound **2** did not show any more growth.

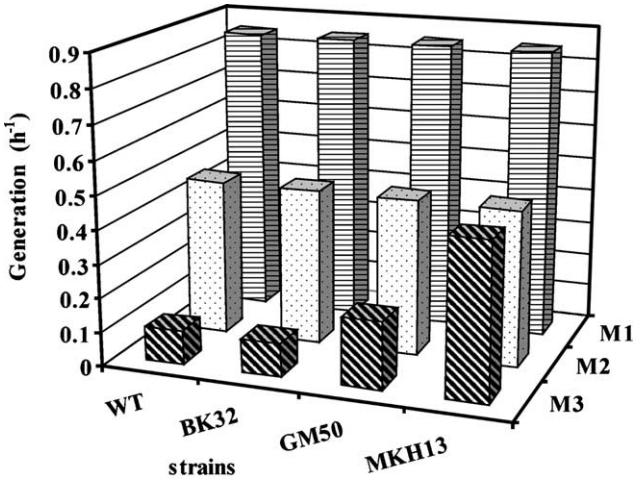


Figure 1. Growth rate of *E. coli* strains on M63 medium without NaCl (**M1**), with added 0.3 M NaCl (**M2**) and with added 0.3 M NaCl + compound **1** (**M3**). WT (wild type MC4100), BK32 (mutant *proP*), GM50 (mutant *proU*), MKH13 (double mutant *proUproP*).

In conclusion we infer that an active transport, through osmoporters, is required for the toxic activity of compounds **1** and **2** and that both growth inhibitors display a differential effect, with only that of **2** being lethal.

To understand why **1** and **2** exerted a differential effect, cultures in mid exponential phase of *E. coli* MC4100 grown in M63 and M63-0.3 M NaCl were supplied with radiolabelled GB analogues **1r** and **2r**;[17] bacterial cells were harvested by centrifugation and concentrated to an $OD_{570} = 10$. A rapid screening of other bacteria grown in their corresponding defined medium was also carried out.

Compounds **1r** and **2r**, ^{14}C-labelled on their C1 carbon were actively imported by the bacterial cells and, as expected, their transport activity was severely enhanced by salt addition to the assay medium. For *E. coli*, analysis of chromatographic data from compound **1** clearly showed that only one labelled solute was present in the bacterial extract (Fig. 2); moreover, no radioactivity was found either in evolved CO_2 or in the insoluble pellet, even after 10 h of incubation. That means the totality of absorbed **1r** remained unchanged in *E. coli*. In contrast, compound **2r** was rapidly metabolised, leading to labelled compounds, among which only dimethylglycine, also known as a demethylated catabolite of glycine betaine, was identified by co-chromatography (Fig. 2). The other part of the molecule, not labelled, produced a yellow colour in the culture medium and could be attributed to 4-nitrobenzaldehyde. The degradation level of **2r** was time-dependent; almost all the substrate had disappeared after 1 h of incubation. Data from other strains clearly demonstrated a behaviour of

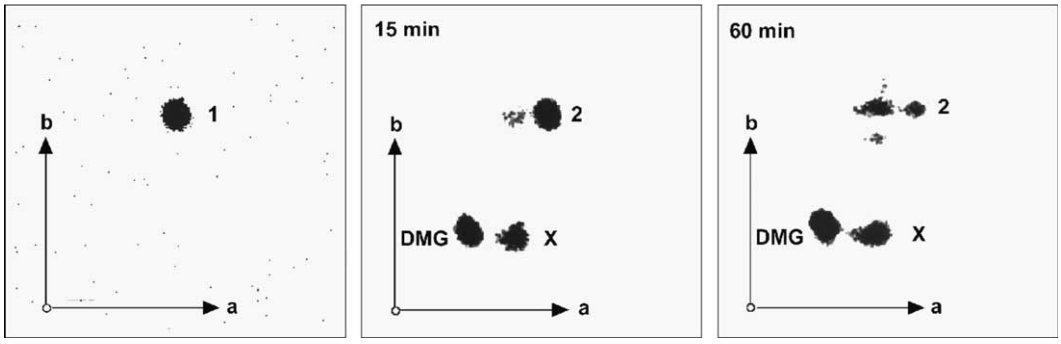


Figure 2. Electronic autoradiography of a 2D-TLC analysis of the soluble fraction of *E. coli* MC4100 cells treated with compounds **1r** (60 min) and **2r** (15 and 60 min). Solvent **a**: *n*-butanol–acetic acid–water (12:3:5); solvent **b**: phenol–ethanol–ammonia (50:50:0.5). X: unknown radiolabelled compound.

both radiolabelled betaines quite similar to that observed in *E. coli*. Interestingly, all the strains sensitive to the treatment must possess at least one transport system equivalent to those of *E. coli*.

Together, our data demonstrate a powerful inhibitory effect of glycine betaine analogues against several bacterial strains belonging to diverse taxonomic groups. The toxic activity is manifest mainly for compounds **1** and **2**. The former displays a bacteriostatic effect whereas compound **2** seems lethal, probably because of the release of a nitrobenzyl aldehyde resulting from its metabolism. Efficient specific transport systems are required for importation of compounds **1** and **2**; they are strikingly activated by elevation of medium osmolality and do allow a high internal accumulation of the toxic analogues, as they do for glycine betaine.

Indeed, such efficient systems could be a useful means to promote penetration of drugs into recalcitrant bacteria. Curiously, very few reports dealing with successful attempts using betaine analogues are available so far.[18] Unfortunately, the mechanism of inhibition exerted by betaine analogues still remains unknown; preparation of resistant mutants (Tn5 insertion) would help to determine the actual target of these inhibitors. In addition, synthesis of new molecules, structurally related to glycine betaine or other osmoprotectants, should be encouraged.

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References and notes

1. Csonka, L. N.; Hanson, A. D. *Annu. Rev. Microbiol.* **1991**, *45*, 569.
2. Booth, I. R.; Higgins, C. F. *FEMS Microbiol. Rev.* **1990**, *6*, 239.
3. Galinski, E. A. *Adv. Microbiol. Physiol.* **1995**, *37*, 272.
4. Cosquer, A.; Pichereau, V.; Pocard, J. A.; Minet, J.; Cormier, M.; Bernard, T. *Appl. Environ. Microbiol.* **1999**, *65*, 3304.
5. Koo, S. P.; Booth, I. R. *Microbiology* **1994**, *140*, 617.
6. Kempf, B.; Bremer, E. *Arch. Microbiol.* **1998**, *170*, 319.
7. Chambers, S. T.; Lever, M. *Nephron* **1996**, *74*, 1.
8. Cosquer, A.; Pichereau, V.; Le Mée, D.; Le Roch, M.; Renault, J.; Carboni, B.; Uriac, P.; Bernard, T. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 4954.
9. Quéau, M.; Renault, J.; Uriac, P.; Corbel, J. C.; Travert, M. F.; Bernard, T.; Donnio, P. Y. *Pathol. Biol.* **2003**, *51*, 516.
10. Lloyd, A. W.; Smith, G.; Oliff, C. J.; Rutt, K. J. *J. Pharm. Pharmacol.* **1992**, *44*, 507.
11. Takeuchi, M.; Mizuno, T.; Shinmori, U.; Nakashima, M.; Shinkai, S. *Tetrahedron* **1996**, *52*, 1195.
12. Structural data for compound **1**. HRMS (LSIMS) $[M+H]^+$ $C_{11}H_{16}NO_2$ calcd 194.1181 found: 194.118 negative mode $[M+Cl]^-$: 228. ^1H NMR (500 MHz, D_2O) 3.26 (s, 6H), 4.03 (s, 2H), 4.75 (s, 2H), 7.55 (m, 5H). ^{13}C NMR (125 MHz, D_2O) 51.36; 61.08; 68.48; 127.16; 129.69; 131.38; 133.21; 167.70.
13. Electrophoresis and chromatography. High voltage paper electrophoresis: a sheet of Whatman 3 MM paper, moistened with 3% formic acid was submitted to an electric field value of 30 V/cm. Extract subsamples were spotted at the anode side and migration was maintained for about 60 min.
2-D TLC on silica gel 60 F254 Merck using as solvent 1, *n*-butanol–acetic acid–water (12:3:5 v/v) and as solvent 2, phenol (80% in water)–ethanol–ammonia (50:50:0.5 v/v).
14. Typical experimental procedure. *Anchorage of dimethylglycine*: dimethylglycine (0.67 g; 6.5 mmol) and HOBT (0.77 g; 6.5 mmol) were stirred for 5 min in anhydrous DMF (5 mL). Then DCI (0.82 g; 6.5 mmol) was added. This solution, after stirring for 1 h at 0 °C and 1 h at rt, was added to a suspension of Wang resin (1 g; 0.65 mmol) in anhydrous DMF (10 mL) in a 25 mL syringe. Washing was achieved as following: DMF, THF, THF/water (50/50), water, THF, anhydrous ether and then the resin was dried under reduced pressure.
Alkylation: to a suspension of a resin in anhydrous DMF (10 mL) 5 equiv (3.25 mmol) of alkylating agent in 2 mL of DMF was added. After stirring at rt for 24 h the same washing procedure was used.
Cleavage: Wang resin (1 g) was allowed to cleave in 6 mL of a 50:50 mixture of TFA and CH_2Cl_2. After evaporation of the solvent, the crude residue was stirred for 30 min in a mixture of 1.5 mL of water and 1.5 mL of CH_2Cl_2. The

aqueous layer was washed twice using 2 mL of CH_2Cl_2 and then evaporated. The solid residue was dried under reduced pressure.

15. (a) Ziegler, E.; Wittmann, H. *Monatsh. Chem.* **1985**, 821; (b) Du Pont de Nemours & Co, U.S. Patent 2359863, **1942**.
16. Crist, D. R.; Jordan, G. J.; Moore, D. W.; Hashmall, J. A.; Borsetti, A. P.; Turujman, S. A. *J. Am. Chem. Soc.* **1983**, *105*, 4136.
17. Radiolabelling assays: typically, a diluted suspension (OD = 1) in either salted or unsalted medium was supplemented with the radioactive substrate. $^{14}CO_2$ was trapped in a concentrated solution of KOH (5 M); the whole soluble metabolites were extracted with 80% ethanol and the insoluble material was gathered in the pellet after centrifugation. The soluble fraction was analysed on a 2D thin-layer, as in Ref. 13. Detection of the radiolabelled compounds was performed using the InstantImager Packard (electronic autoradiography).
18. Peddie, B. A.; Wood, J. E.; Lever, M.; Happer, D. A.; De Zwart, F.; Chambers, S. T. *Antonie van Leeuwenhoek* **2003**, *83*, 175.