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SYNTHESIS OF P-TRIAZINYLPHOSPHONIUM SALTS – HYBRID MOLECULES WITH POTENTIAL ANTIMICROBIAL ACTIVITY*

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Abstract: The new hybrid drugs combining in a single molecule triazine ring attached to phosphonium salt were prepared and their bactericidal activity against Gram-positive bacteria *Staphylococcus aureus* ATCC43300 (MRSA – methycyline resistant *Staphylococcus aureus* strain) two Gram-negative bacteria *Escherichia coli* CCUG31997 serotype O153 (EPEC – enteropathogenic *Escherichia coli* strain), *Proteus mirabilis* 1784 (MDR – multidrug resistant clinical strain) were determined using microdilution method.

Keywords: hybrid drugs, 1,3,5-triazine, phosphonium salt, phosphonium biocide

The hybrid drugs, just as their name implies, combine two drugs in a single molecule with the goal of creating a chemical entity more medically effective than its individual components. A major driving force in advancing this approach is to overcome the development of resistance in the target population. In most of the cases, in such hybrids both drug-like fragments have independent modes of action that make the emergence of drug resistance less likely. Moreover, in several cases the collected data provide a conclusive molecular mechanism for the amplification of effects of fragments or even the extraordinary biological effects not attributed to any of the individual partner of the hybrid construct (1). In the design of new compounds, development of hybrid molecules through the combination of different pharmacophores in one structure may lead to compounds with increased antimicrobial activity. Problems of multi-drug resistant microorganisms have reached an alarming level in many countries around the world. A numbers of recent clinical reports describe the increasing occurrence of *S. aureus* and other antibiotic-resistant human pathogenic microorganisms in United State and European countries. Infections caused by those microorganisms pose a serious challenge to the medical community and the need for an effective therapy has led to a search for novel antimicrobial agents. Exploitation of these molecules should allow us to rapidly discover new biologically active compounds across a broad range of therapeutic areas in a shorter time scale.

Among the compound having good antimicrobial properties, s-triazine derivatives (particularly melamine derivatives) constitute an important class of compounds possessing diverse pharmacological activities including broadly active as herbicidal (2) and antimicrobial (3). Some are also used for the treatment of HIV infection (4). Several workers investigated the s-triazine nucleus in the scope of potential therapeutic agents for diseases due to bacteria (5) cancer (6) antitumor (7) and malaria (8) .

It is also well known, that positively charged compounds such as long chain quaternary ammonium compounds exert antibacterial activity against both Gram-positive and Gram-negative bacteria, as well as against other pathogenic species of fungi and protozoa (9). Quaternary ammonium salts belong to group of hard antibacterial agents (10) that are biologically active and non-metabolizable *in vivo* (soft drugs (11) are defined as drugs, which are characterized by predictable and controllable *in vivo* destruction to form non-toxic products after they have achieved their therapeutic role). Thus, although the

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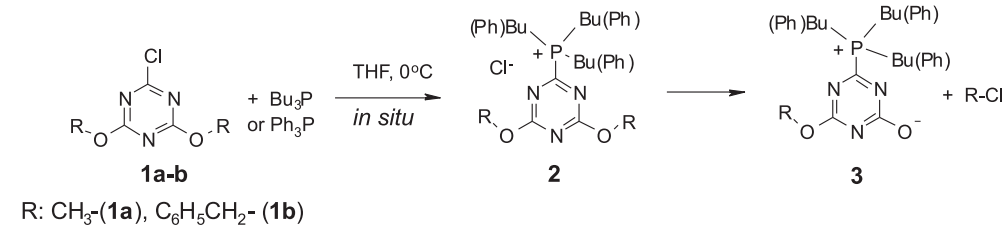


Scheme 1. Synthesis of P-triazinylphosphonium salts

soft analogs have been shown to possess antibacterial activity *in vitro*, it is likely that their *in vivo* activity will be hampered by their chemical instability. It has been found that quaternary phosphonium salts also showed hard antibacterial activity (12).Various phosphonium salts possessing single or double alkyl chains of various lengths (C10 to C18) were prepared as cationic biocides, and their antimicrobial activities against 11 typical strains of microorganisms including methicillin-resistant *Staphylococcus aureus* (MRSA) were evaluated. The phosphonium salts with long alkyl chains were found to show high levels of antimicrobial activity. Their activities depended strongly on the molecular structure, and a correlation between antimicrobial activity and molecular structure was observed. In the alkyltrimethylphosphonium salts, the bactericidal activity against *S. aureus* and *Escherichia coli* increased with increasing alkyl chain length. In contrast, the bactericidal activity of dialkyldimethylphosphonium salts was found to decrease as the chain length of the substituents increased. It is significant that the phosphonium biocide containing double decyl groups exhibited the broadest spectrum of activity against microorganisms tested and showed the greatest bacteriostatic activity against MRSA. It was observed that the phosphonium salts showed an advantage over the corresponding ammonium salts in bactericidal activity and killing rate.

Therefore, these observations prompted us to synthesize new 1,3,5-triazine derivatives which were attached with quaternary phosphonium salts. Then, the synthesized compounds were tested against Gram-positive bacteria and Gram-negative bacteria using microdilution method.

EXPERIMENTAL

Thin layer chromatography (TLC) was carried out on SiO_2 (Merck; 60 F_{254}) and spots were located with: UV light (254 and 366 nm) and 1% ethanolic 4-(4-nitrobenzyl)pyridine (NBP). Melting points were determined on a Büchi apparatus, model 510. ^{1}H-NMR, ^{31}P-NMR, spectra were recorded on a Bruker Avance DPX 250 (250 MHz) spectrometer. Chemical shifts (ppm) are relative to TMS used as an internal standard. The multiplicities were marked as s =singlet, d = doublet, t = triplet, q = quartet, qu = quintet, m = multiplet.

Reaction of 2-chloro-4,6-dimethoxy-1,3,5-triazine (1a) with tributylphosphine

To intensively stirred solution of 2-choro-4,6-dimethoxy-1,3,5-triazine (CDMT, **1a**) (1.75 g, 10 mmole) in THF (30 mL) cooled down to 0°C Bu_3P (2.5 mL, 10 mmole) was added. Stirring was continued until disappearance of **1a** that was confirmed by using NBP test (about 24 h). The precipitate was filtered, washed carefully with THF (3 × 5 mL) and dried in vacuum desiccator to constant weight yielding 4-methoxy-6-(tributyl-λ^5-phosphanyl)-[1,3,5] triazin-2-ol (**3a1**), (1.22 g, yield 37%), mp = 166–168°C. ^{31}P-NMR (CD_3CN, δ, ppm): 26.89. ^{1}H-NMR (CD_3CN, δ, ppm): 0.95 (t, 9H, J = 7 Hz, -CH_2-CH_3), 1.42–1.63 (m, 12H, -CH_2-CH_2-CH_3), 2.28–2,40 (m, 6H, -P-CH_2-), 3.95(s, 3H, -OCH_3). Analysis: for $C_{16}H_{31}N_3O_2P$ (328.42): calcd.: C 58.52, H 9.51, N 12.79%; found: C 56.23, H 9.30, N 12.82%.

Reaction of 2-chloro-4,6-dimethoxy-1,3,5-triazine (1a) with triphenylphosphine

Starting materials: 2-choro-4,6-dimethoxy-1,3,5-triazine (CDMT, **1a**) (1.75 g, 10 mmole), PPh_3 (2.62 g, 10 mmole), THF (30 mL). Product: 4-methoxy-6-(triphenyl-λ^5-phosphanyl)-[1,3,5]triazin-2-ol (**3a2**) (1.55 g, yield 40%), mp = 247–260°C. ^{31}P-NMR (CD_3CN, δ, ppm): 9.78. ^{1}H-NMR (CD_3CN, δ, ppm): 3.93 (s, 3H, -OCH_3), 7.62–7.85 (m, 15H, -P-$(C_6H_5)_3$). Analysis: for $C_{22}H_{18}N_3O_2P$ (387.38) calcd.: C 68.21, H 4.68, N 10.85%; found: C 67.93, H 4.72, N 10.82%.

Reaction of 2-chloro-4,6-dibenzyloxy-1,3,5-triazine (1b) with tributhylphosphine

Starting materials: 2-chloro-4,6-dibenzyloxy-1,3,5-triazine (**1b**) (3.30 g, 10 mmole), Bu_3P (2.5 mL, 10 mmole), THF (30 mL). Product: 4-benzyloxy-6-(tributyl-λ^5-phosphanyl)-[1,3,5]triazin-2-ol (**3b1**) (1.33 g, yield 33%), m.p. 166–169°C. ^{31}P-NMR (CD_3CN, δ, ppm): 26.91. ^{1}H-NMR (CD_3CN, δ, ppm): 0.92 (t, 9H, J = 8 Hz, -CH_2-CH_3), 1.35–1.50 (m, 12H, -CH_2-CH_2-CH_2-), 2.19–2.38 (m, 6H, -CH_2-CH_2-), 5.53 (s, 2H, -O-CH_2-), 7.28–7.49 (m, 5H, CH_2-C_6H_5). Analysis: for $C_{22}H_{34}N_3O_2P$ (403.51) calcd.: C 65.49, H 8.49, N 10.41%; found: C 65.55, H 8.38, N 10.39%.

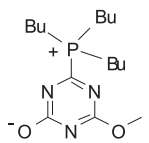
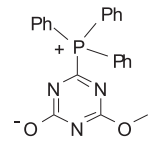
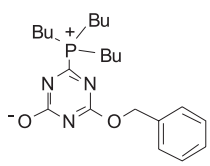
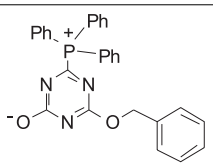
Reaction of 2-chloro-4,6-dibenzyloxy-1,3,5-triazine (1b) with triphenylphosphine

Starting materials: 2-chloro-4,6-dibenzyloxy-1,3,5-triazine (**1b**) (3.30 g, 10 mmole), PH_3P (2.62 g, 10 mmole), THF (30 mL). Product: 4-benzyloxy-6-(triphenyl-λ^5-phosphanyl)-[1,3,5]triazin-2-ol (**3b2**) (1.33 g, yield 62%), m.p. 224–227°C. ^{31}P-NMR (CD_3CN, δ, ppm): 9.55. ^{1}H-NMR (CD_3CN, δ, ppm): 5.55 (s, 2H, -O-CH_2-), 7.28–7.49 (m, 5H, -CH_2-C_6H_5), 7.62–7.85 (m, 15H, -P-$(C_6H_5)_3$). Analysis: for $C_{28}H_{22}N_3O_2P$ (463.48) calcd.: C 72.56, H 4.78, N 9.07%; found: C 72.73, H 4.72, N 9.02%.

Pharmacology

Minimal inhibitory concentrations (MIC) of substances were determined using microdilution method (13) on Miller-Hinton medium with following strains: *Escherichia coli* CCUG31997 serotype O153 (EPEC – enteropathogenic *Escherichia coli* strain), *Staphylococcus aureus* ATCC43300 (MRSA – methicillin resistant *Staphylococcus aureus* strain) and *Proteus mirabilis* 1784 (MDR – multidrug resistant clinical strain). Bacterial strains were grown on Miller-Hinton medium at 37°C. Overnight cultures of bacteria were diluted to concentration about 10^{-6} cfu/mL (for *E. coli* and *P. mirabilis*) or

Table 1. Reaction of triazine derivatives **1a-b** with Bu_3P and PPh_3.

Triazine derivative	Phosphine	Product	Yield (%)
1a	Bu_3P	 **3a1**	37
	PPh_3	 **3a2**	40
1b	Bu_3P	 **3b1**	33
	PPh_3	 **3b2**	62

about 10^{-5} cfu/mL (for *S. aureus*). Dilutions were used for the test.

Inhibition test was performed on sterile microtitration 96-well plate with U-shaped bottom.

Four tested substances (**3a1, 3a2, 3b1, 3b2**) were dissolved in 99% ethanol to give concentrations 5 mg/mL. Then, the ethanol solutions of substances were diluted in Miller-Hinton medium to concentrations 0.4 mg/mL. The solutions of substances (0.1 mL) were then logarithmically diluted from 0.2 mg/mL to 0.015 mg/mL. To each well with appropriate dilution of inhibitory substances bacterial cultures were added. Microplates were incubated overnight at 37°C; after centrifugation of cultures in plates (2000 × g, 20 min) the sedimentation of bacterial cells were observed.

As the control, bacterial growth on Miller-Hinton medium with appropriate concentration of ethanol was used. Each determination was performed in triplicate.

Minimal bactericidal concentration (MBC) was determined on the plate with Miller-Hinton agar. From wells where bacterial sediment were not observed, 0.1 mL of culture was sampled on agar plate. After overnight incubation in 37°C a number of cfu (colony forming unit) was determined on each plate.

RESULTS AND DISCUSSION

As mentioned above, the appropriate modification of 1,3,5-triazine ring system could lead to the development of numerous novel and diverse target-specific drugs. The structural motif common to all of these biologically active compounds is the presence of triazine ring with appropriate substituents. Such a multi-target dual action drugs with two dissimilar drug molecules combined together by direct linking of the two molecular entities are already known and found to be useful tools in the therapy of other complex diseases such as cardiovascular and inflammatory diseases and might be used as an antibacterial agents useful in the treatment of drug resistant pathogens (14).

Therefore, while designing the structure of new hybrid antibacterial agents we attempted to attach well known biocidal motif of quaternary phosphonium salts to triazine ring. In order to prepare such constructs we made use of the observation (15) that 2-chloro-4,6-disubstituted-1,3,5-triazines (**1**) in reaction with tertiary amine gave appropriate N-triazinylammonium chlorides. Application of tertiary phosphine in place of tertiary amines gave appropriate P-triazinylphosphonium salts (**3**). Quaternization process using Bu_3P and PPh_3 and **1** proceeded substantially less readily then analogous reaction with tertiary amines.

Moreover, it has been found, that quaternization at phosphorus atom is accompanied by a new, additional process of dealkylation with elimination of the substituent from triazine ring. This reaction course has been found unexpected, because in case of N-triazinylammonium salts, dealkylation (if observed) proceeded with removing of substituent from ammonium nitrogen.

The P-triazinylphosphonium salts **3** obtained were tested as antibacterial agents useful in the treatment of drug resistant pathogens. The values of minimal inhibitory concentrations (MIC) of salts **3** were determinated using microdilution method on Miller-Hinton medium with the following strains: *Escherichia coli* CCUG31997 serotype O153 (EPEC – enteropathogenic *Escherichia coli* strain), *Staphylococcus aureus* ATCC43300 (MRSA – methicillin resistant *Staphylococcus aureus* strain) and *Proteus mirabilis* 1784 (MDR – multidrug resistant clinical strain). For all four P-triazinylphosphonium salts **3** MIC was higher than 50 μg/mL. Growth of the *S. aureus* ATCC43300 in the presence of substance **3b2** at concentration of 0.2 mg/mL was not observed. Analysis of MBC indicated that **3b2** was not bactericidal, but reduced the number of *S. aureus* ATCC43300 in culture up to 99%. For another bacterial strains tested with four substances no inhibition effects were observed (16).

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REFERENCES

1. Hulsman N., Medema J.P., Bos C., Jongejan A., Leurs R., Smit M.J., de Esch I.J.P., et al.: J. Med. Chem. 50, 2424 (2007).
2. (a) Kaswala P.B., Chikhalia K.H., Shah N.K., Patel D.P., Patel D.H., Mudaliar G.V.: ARKIVOC XI, 326 (2009); (b) Garmouna M., Blanchoud H., Teil M., Blanchard M., Chevreuil M.: Water Air Soil Pollut. 132, 1 (2001).
3. Jain S., Bhambi D., Sharma, R., Talesara L.: Ind. J. Pharm. Sci. 69, 28 (2007).

4. (a) Klenke B., Stewart M., Barett M.P., Brun R., Gilbert I.H.: J. Med. Chem. 44, 3440 (2001); (b) Patel R.B., Chikhalia K.H., De Clercq E.: J. Braz. Chem. Soc. 18, 2 (2007).
5. (a) Srinivas K., Shrinivas U., Rao V.J., Bhanuprakash K., Chishore K.H., Murty U.S.N.: Bioorg. Med. Chem. Lett. 15, 1121. (2005); (b) Lubbers T., Angechm P., Gumunder H., Herzig S., Kulhanek J.: Bioorg. Chem. Lett. 3, 821 (2000); (c) Desai A.D., Mahajan D.H., Chikhalia K.H.: Ind. J. Chem. 46B, 1169 (2007); (d) Srinivas K., Srinivas U., Bhanuprakash K., Harakishore K., Murthy U.S.N., Rao V.J.: Eur. J. Med. Chem. 41, 1240 (2006).
6. (a) Alteramn M., Samulsson B.: J. Med. Chem. 41, 3782 (1998); (b) Katritzky A.R., Rogovoy b.V., Vvedensky V.Y., Hebert N., Forood B.: J. Org. Chem. 66, 6797 (2001); (c) Mayumi O., Kawahara N.; Santo Y.: Cancer Res. 56, 1512 (1996).
7. (a) Brozowski Z., Gdaniec M.: Eur. J. Med. Chem. 35, 1053 (2000); (b) Shinichi Y., Yasuhisa F., Ichiro K., Hisashi Y., Toshiyuki M., et al.: J. Natl. Cancer Inst. 98, 545 (2006).
8. Youngyuth Y., Tirayut V., Netnapa, C., Sumalee K., Gordon L.: J. Med. Chem. 43, 2738 (2000).
9. Petrocci A.N.: in Disinfection, Sterilization and Preservation.; Block S.S., Ed., p. 309, Lea & Febiger, Philadelphia 1983.
10. (a) Thorsteinsson T., Loftsson T., Masson M.: Curr. Med.Chem. 10, 1129 (2003); (b) Thorsteinsson T., Masson M., Kristinsson K.G., Hjálmarsdóttir M.A., Hilmarsson H., Loftsson T.: J. Med. Chem. 46, 4173 (2003).
11. Bodor N., Kaminski J.J., Selk S.: J. Med. Chem. 23, 469 (1980).
12. Kanazawa A., Ikeda T., Endo T.: Antimicrob. Agents Chemother. 38, 945 (1994).
13. Kelly L., M., Jacobs M.R., Appelbaum P.C.: J. Antimicrob. Chemother. 43, 707 (1999).
14. Jian H., Anderson M.H., Shi W., Eckert R.: Int. J. Antimicrob. Agents 33, 532 (2009).
15. Kamiński Z.J.: Synthesis 10, 917 (1987).

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