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NEW *N*-SUBSTITUTED 7-AMINOCEPHALOSPORANIC ACID DERIVATIVES AS POTENTIAL AGENTS AGAINST *STREPTOCOCCUS PNEUMONIAE*. SYNTHESIS AND *IN VITRO* ACTIVITY

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Abstract. The synthesis and the antimicrobial properties of a new series of cephalosporinic β-lactam antibiotics is described. The data reported in the present paper show the potential of this type of substituted cephalosporins as new anti Gram-positive antibiotic drugs. In fact, all compounds tested showed a good *in vitro* antibacterial activity against the most relevant Gram-positive pathogens including resistant species that currently represent unmet medical need. On the contrary, the new synthesized compounds were found to be completely devoid of any activity on Gram-negative bacteria up to a concentration of the single agent of 128 μg/ml.

Upper and lower respiratory tract infections are a very frequent cause of illness in patients population of all ages in all countries worldwide. Amongst the organisms causing lower respiratory tract infections (LRTIs) and upper respiratory tract infections (URTIs) both *Streptococcus pneumoniae* and *Streptococcus pyogenes* are the main pathogens. The worldwide incidence of infectious caused by pneumococci resistant to penicillin G and other class of antibiotics has increased at an alarming rate during the last five years. The main foci of penicillin-resistant pneumococci are currently South Africa, Spain and Eastern Europe, but the spread of penicillin-resistant clones demonstrates the capability of these strains to spread rapidly throughout the world. A recent epidemiological survey by Doern and co-workers[1] has documented erythromycin A resistance rates of 20% and 49% in penicillin-intermediate resistant and resistant strains, respectively.

Penicillins, cephalosporins and macrolides have traditionally been used to eradicate these pathogens in both upper and lower RTIs. In recent years, however, the advent of β-lactamase production, alteration in penicillin binding proteins, macrolide resistance and recognition of cross resistance among different antibacterial classes has led to a re-evaluation of existing antibiotics and a search for newer highly active compounds that display little or no cross resistance, with a narrow spectrum of action to niche-resistant pathogens. Considering that among β-lactams the combination amoxicillin/clavulanate and ceftriaxone are the oral and injectable drugs of choice, respectively, to treat infections caused by resistant pneumococci, our work was aimed at the synthesis of novel cephalosporins with improved *in vitro* antibacterial activity against RTI Gram-positive pathogens.

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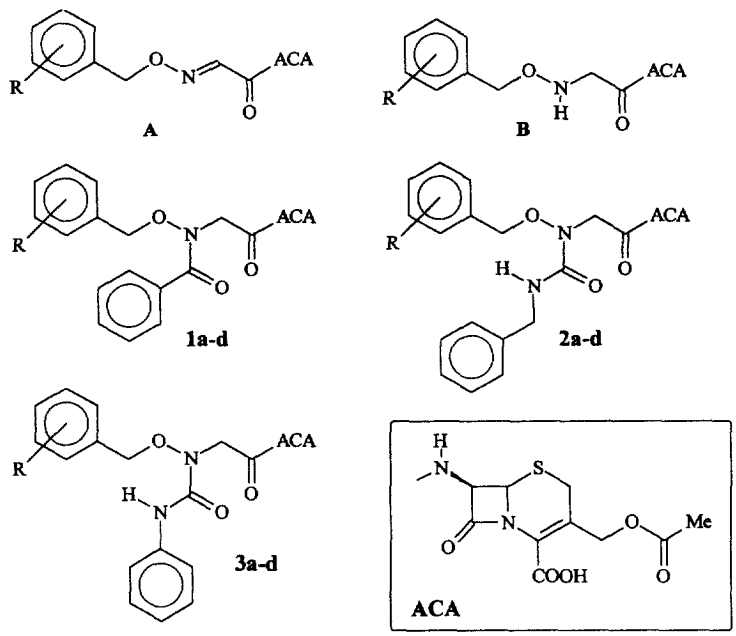


a, R = H; **b**, R = *o*-Cl; **c**, R = *m*-Cl; **d**, R = *p*-Cl

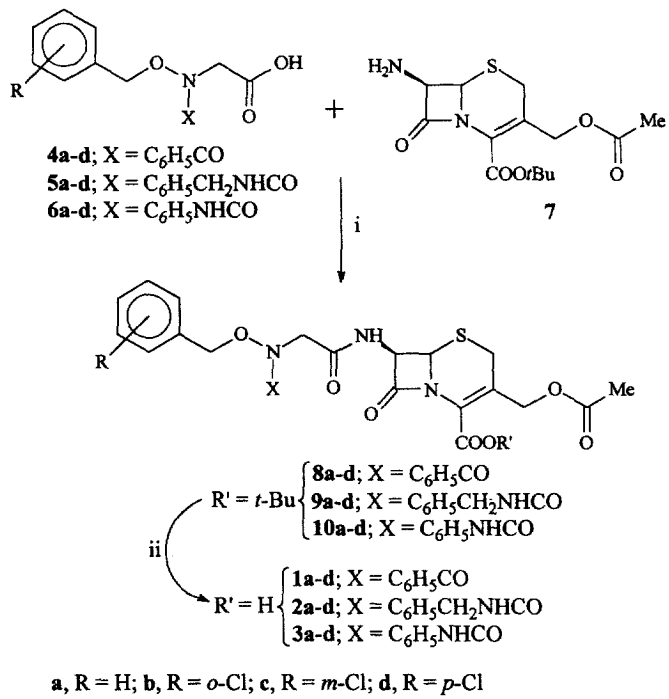
In previous studies aiming at investigating the effects on the antimicrobial properties induced by certain structural modifications on the amidic side chain linked to the C(7) carbon of the cephalosporinic nucleus, we found that type **A** compounds, in which this side chain contains an aryl substituted [(methyloxy)imino]methyl moiety (MOIMM), showed an appreciable activity directed only towards Gram positive microorganisms.[2] The further replacement of the MOIMM with a more polar hydroxylaminoethereal portion lead to type **B** compounds possessing practically identical antimicrobial features.[3]

QSAR studies in the field of cephalosporinic β-lactam antibiotics demonstrated that an increase in the steric hindrance and/or lipophilicity of the amidic side chain is able to positively influence the antimicrobial activity against Gram-positive microorganisms.[4] On the basis of this finding we thought to verify whether structural modifications on the amidic side chain of type **B** compounds able to increase both the lipophilicity and the steric hindrance might lead to an improvement of the activity against Gram-positive bacteria and more specifically against RTI pathogens.

We here report the synthesis and the antimicrobial properties of a new series of cephalosporinic β-lactam antibiotics (compounds **1a-d, 2a-d, 3a-d**) in which the hydroxylaminic nitrogen of type **B** compounds has been replaced with benzamidic (**1a-d**), benzylureic (**2a-d**) and phenylureic (**3a-d**) functions.

Chemistry. Compounds **1a-d, 2a-d, 3a-d** were prepared as outlined in Scheme 1. Treatment of the N-(benzyloxy)glycine derivatives **4a-d, 5a-d, 6a-d**[5] with an equimolar amount of 7-ACA protected as *t*-butyl ester (7)[2] in the presence of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride as the dehydrating agent gave the corresponding β-lactam esters **8a-d, 9a-d, 10a-d**, [6] which were purified by column chromatography. Hydrolysis of **8a-d, 9a-d, 10a-d** with trifluoroacetic acid and anisole afforded the desired compounds **1a-d, 2a-d, 3a-d**.[6]

Scheme 1



a, R = H; **b**, R = *o*-Cl; **c**, R = *m*-Cl; **d**, R = *p*-Cl

Reagents and conditions. (i) 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (1.2 eq.), CH_2Cl_2 an., r.t., 12h; yields from 52% to 80%. (ii) Trifluoroacetic acid (18 eq.), anisole (1.3 eq.), CH_2Cl_2 an., 0 °C, 24h; yields from 62% to 46%.

Results and Discussion. The *in vitro* antibacterial activity of the synthesised cephalosporins against common Gram-positive pathogens[7] is summarised in Table 1. A comparison with marketed oral and injectable antibiotics is also reported.[8] Data are expressed as Minimum Inhibitory Concentration (MIC) values (μg/ml).[9] Compounds **1a-d, 2a-d** and **3a-d** were also tested against Gram-negative bacteria[10], but they were found to be completely devoid of any activity up to a concentration of the single agent of 128 μg/ml.

Table 1. *In vitro* antibacterial activity of the synthesised compounds against Gram-positive pathogens in comparison with reference antibiotics.

	MIC (μg/ml)[a]													
Compound	*S. a.*[b] 663E Pen-S[e]	*S. a.* 3072 Pen-S	*S. a.* 3849 Pen-S	*S. a.* 853 Pen-R[f]	*S. a.* 2672 Pen-R	*S. a.* 2619 Pen-R	*St. p.*[c] 3512 Pen-S	*St. p.* 3272 Pen-S	*St. p.* 3273 Pen-S	*St. p.* 4629 Pen-R	*St. p.* 4630 Pen-R	*St. p.* 4635 Pen-R	*St. p.* 4636 Pen-R	*St. py.*[d] Ery-R
1a	0.5	1	0.5	1	8	2	0.03	0.06	0.06	8	8	8	16	0.25
1b	1	1	0.5	1	8	4	0.25	0.25	0.25	16	16	16	32	0.5
1c	0.5	0.5	0.5	0.5	4	1	0.06	0.06	0.12	8	8	1	2	0.25
1d	0.5	0.5	1	2	8	2	0.12	0.12	0.12	8	8	8	16	0.12
2a	0.5	1	1	1	2	1	0.25	0.12	0.5	16	16	4	32	0.25
2b	0.5	1	0.5	1	4	1	0.25	0.5	0.5	16	16	4	64	0.25
2c	1	1	0.5	1	2	2	0.25	0.25	0.5	8	8	4	32	0.25
2d	0.5	1	0.5	1	2	1	0.25	0.25	0.5	8	8	4	32	0.12
3a	0.5	1	0.5	1	4	2	0.25	0.12	0.25	8	8	8	16	0.12
3b	0.5	0.5	0.5	2	2	1	0.12	0.25	0.25	8	8	4	8	0.12
3c	0.5	0.5	0.5	1	2	1	0.12	0.06	0.12	4	4	2	4	0.12
3d	0.5	0.5	0.5	1	4	1	0.06	0.12	0.25	8	4	4	16	0.06
Penicillin G	≤ 0.5	≤ 0.5	≤ 0.5	64	64	64	≤ 0.5	≤ 0.5	0.5	4	4	8	8	N.D.[h]
[g]Amox/Clav	0.25	0.5	0.25	2	1	2	≤ 0.015	≤ 0.015	≤ 0.015	4	2	4	4	0.06
Cefuroxime	1	2	1	2	2	2	0.06	0.06	0.25	32	16	32	16	0.06
Cefaclor	1	4	1	64	64	16	1	1	0.5	32	16	16	16	0.5
Ceftriaxone	2	4	2	4	4	4	0.03	0.03	0.03	4	2	1	1	0.06
Cefpirome	1	1	1	2	2	2	0.06	0.06	0.06	2	1	≤ 0.5	≤ 0.5	0.03

[a]Minimum Inhibitory Concentration. [b]*Staphylococcus aureus*. [c]*Streptococcus pneumoniae*. [d]*Streptococcus pyogenes* Erythromycin-resistant. [e]Penicillin-sensitive. [f]Penicillin-resistant. [g]Amoxicillin/Clavulanate. [h]Not Determined.

In contrast they displayed interesting antibacterial activity against Gram-positive strains, definitely better than that previously shown by type **A** and **B** compounds.[2,3]

The activity of the tested compounds against penicillin-sensitive (Pen-S) and penicillin-resistant (Pen-R) *Staphylococcus aureus* was comparable to that of amoxicillin/clavulanate combination, second- and fourth-generation cephalosporins, cefuroxime and cefpirome, respectively, but was superior to ceftriaxone (a third-generation cephalosporin); penicillin G. Methicillin-resistant *Staphylococcus aureus* (MRSA) strains were completely resistant to all compounds tested (data not shown). Although the majority of compounds tested

showed comparable antibacterial activity against Pen-S and Pen-R *Staphylococcus aureus*, a little cross resistance with other β-lactams was observed for **Ia, Ib** and **Id.** This was probably due to the hydrolysis of the compounds mediated by β-lactamases produced by the bacterial strains. All Pen-S *Streptococcus pneumoniae* were sensitive to the β-lactams tested showing MIC values ≤ 0.5 μg/ml. Cefaclor was the least active antibacterial agent tested. The susceptibility of Pen-R *Streptococcus pneumoniae* to β-lactams was inferior than that observed against Pen-S *Streptococcus pneumoniae* because of the lower affinity towards the enzymatic β-lactam target namely penicillin binding protein (PBP).[11] The antibacterial activity of new derivatives against four strains of *Streptococcus pneumoniae*, highly resistant to penicillin G, was comparable to the activity of the therapeutic agents amoxicillin/clavulanate, ceftriaxone and cefpirome currently used to eradicate resistant-pneumococci, as oral or injectable drugs.

As expected, the susceptibility of an erythromycin resistant *Streptococcus pyogenes* strain to all β-lactams tested was very high (MIC ≤ 0.25 μg/ml) because the mechanisms of resistance to macrolides are completely different from those relevant for β-lactam antibiotics.

Conclusions. The good and selective anti Gram-positive activity, including resistant species, shown by compounds **Ia-d, 2a-d** and **3a-d** in which the hydroxylaminic nitrogen of type **B** compounds was replaced with benzamidic **(Ia-d),** benzylureic **(2a-d)** and phenylureic **(3a-d)** functions confirm how an increase in the steric hindrance and/or the lipophilicity of the amidic side chain is able to positively influence the antibacterial properties against Gram-positive pathogens. The data reported in the present paper show the potential of this type of substituted cephalosporins as new anti Gram-positive antibiotic drugs. In fact, all compounds tested showed a good *in vitro* antibacterial activity against the most relevant Gram-positive pathogens including resistant species that currently represent unmet medical need.

The overall antibacterial activity of the cephalosporinic derivatives here tested is comparable to that observed for the amoxicillin/clavulanate combination, ceftriaxone and cefpirome (third- and fourth-generation cephalosporins) and superior to that showed by second-generation cephalosporins (cefaclor and cefuroxime). On the light of the results obtained, these molecules could be explored as novel β-lactams with anti Gram-positive activity including resistant pathogens, although further investigations related either to the elucidation of their mechanism of action against penicillin-resistant *Streptococcus pneumoniae* or of their pharmacokinetic properties, in order to study the efficacy of these molecules in animal models of infection, are needed.

References and Notes

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3. Gentili, D.; Macchia, M.; Menchini, E.; Nencetti, S.; Orlandini, E.; Rossello, A.; Broccali, G.; Limonta, D. *Il Farmaco* **1998**, submitted.
4. Neu, H.C. In *The Chemistry of β-Lactams*; Page, M.I., Ed.; Chapman & Hall, 1992; pp 109-118.
5. Compounds **4a, 5a** and **6a** were prepared as previously reported (Macchia, M.; Barontini, S.; Martinelli, A.; Menchini, E.; Nencetti, S.; Orlandini, E.; Romagnoli, F. *Il Farmaco* **1998**, *53*, 369). Compounds **4b-d, 5b-d, 6b-d** were prepared in an analogous manner as **4a, 5a** and **6a**.
6. Analytical and spectral data of compounds **8a-d, 9a-d, 10a-d** and **1a-d, 2a-d, 3a-d** were in accordance with the structure assigned. In particular ^{1}H NMR spectra of all compounds showed a dd with a chemical shift ranging from 5.70 ppm to 5.88 ppm attributable to the H_7 proton.
7. The Gram-positive strains used in the present work. were clinical isolates and belonged to the Culture Collection of GlaxoWellcome S.p.A. (Verona, Italy). Strain identification and pattern of antibiotic resistance were carried out by using the Vitek apparatus (BioMerieux, Milan, Italy). Each strain was maintained as lyophilised culture and was sub-cultured twice on Blood Agar Base (BBL, Cockeysville, MD) just before use.
8. Penicillin G, cefuroxime, cefaclor, ceftriaxone, cefpirone and amoxicillin/clavulanate combination were obtained from commercial sources.
9. All compounds tested were daily prepared in 0.1 mM sodium phosphate buffer, pH 7.4. Minimal inhibitory concentration (MIC) was determined according to the technical procedure recommended by the National Committee for the Clinical Laboratory Standards (1997. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically - Fourth edition. Approved Standards M7-A4. National Committee for Clinical Laboratory Standards, Villanova, Pa). Serial doubling dilution, in appropriate growth medium, was prepared from each antibiotic solution. The bacterial inoculum was prepared by making a direct saline suspension of isolated colonies from an overnight agar plate. The suspension was adjusted to match the 0.5 McFarland turbidity standard and diluted in sterile broth to obtain a final inoculum of about 5.10^5Colony Forming Unit/ml in the microtiter plate. The plates were inoculated using a replicating device (Dynatech AM80, Molecular Devices, U.S.A.). In the case of *S. pneumoniae* and *S. pyogenes* strains Tood Hewitt media (BBL, Cockeysville, MD) were used and the Minimum Inhibitory Concentration (MIC) value was recorded after 24h of incubation at 35°C in 5 % CO_2 atmosphere. The MIC was defined as the lowest concentration that resulted in no visible growth after 20 hours of incubation at 35°C.
10. *Escherichia coli*, *Pseudomonas aeruginosa*, *Haemophylus influenzae* and *Bacteroides fragilis*, from the *GlaxoWellcome* S.p.A. (Verona, Italy) Bacterial Culture Collection were used as the Gram-negative reference strains.
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