Novel β-Lactam Antibiotics Synthesized by Amination of Catechols Using Fungal Laccase[1)]

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Novel cephalosporins, penicillins, and carbacephems were synthesized by amination of catechols with amino-β-lactams like cefadroxil, amoxicillin, ampicillin and the structurally related carbacephem loracarbef using laccase from *Trametes* sp. All isolated monoaminated products inhibited the growth of several Gram positive bacterial strains in the agar diffusion assay, among them methicillin-resistant *Staphylococcus aureus* strains and vancomycin-resistant Enterococci. Observed differences in the cytotoxicity and *in vivo* activity in a "*Staphylococcus*-infected, immune suppressed mouse" model are discussed.

Key words laccase; catechol; biotransformation; β-lactam antibiotic; resistance

In preceding papers, we discussed the laccase-catalyzed amination of 2,5-dihydroxybenzoic acid derivatives with aminopenicillins and aminocephalosporins.[2,3)] The motive for our work was the global issue with resistant pathogenic bacteria, *e.g. Streptococcus pneumoniae* strains[4—7)] and *Staphylococcus* strains, against currently available β-lactam antibiotics.[8—11)] Aminated products like 2-(3,6-dioxocyclohexa-1,4-dienylamino)-2-phenyl-acetylamino-penicillanic acids and 2-(3,6-dioxocyclohexa-1,4-dienylamino)-2-phenyl-acetylamino-cephalosporanic acids[2,3)] inhibited the growth of several Gram positive bacterial strains and protected mice against an infection with *Staphylococcus aureus*. After amination the 2,5-dihydroxybenzoic acid derivatives were units of the C-7 substituent of the β-lactam antibiotics.

The possibility of utilizing catechol units as an element of the C-7 substituent of penicillins and cephalosporins was investigated for a long time.[12—17)] Through the *ton*B dependent iron transport mechanism,[18)] the use of catechol substituted β-lactams was known to increase the drug's penetration into the bacterial cell walls. Thus, several catechol-substituted β-lactams have been synthesized and showed good antimicrobial activities.[19—23)]

As a consequence of the promising activity of the catechol substituted β-lactams on one hand and the novel laccase-synthesized β-lactams on the other hand, we got interested in laccase-catalyzed amination of catechols with aminopenicillins and aminocephalosporins.

In this study, we have employed laccase from *Trametes* sp. to derivatize amino-β-lactams and to couple them both with catechol and with substituted catechols. The novel β-lactams were structurally characterized as new coupling products inhibiting the growth of several Gram positive bacterial strains in the agar diffusion assay, among them methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant Enterococci. The cytotoxicity of the new compounds and the effectiveness in a "*Staphylococcus*-infected, immune suppressed mouse" model are discussed.

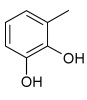
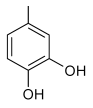
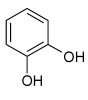
Results and Discussion

Biotransformation of Amino-β-lactams by Laccases

Laccase-catalyzed reaction between catechols (substrates **1a** to **1c**) on one hand and the amino-β-lactams cefadroxil **2a**, amoxicillin **2b**, ampicillin **2c**, and the structurally related carbacephem loracarbef **2d** on the other hand led to cross coupled products (Table 1).

3-Methylcatechol and amino-β-lactams were consumed after an incubation time of 1.5 h, and monoaminated products (**3a** to **3d**) were detectable by high-performance liquid chromatography (HPLC). If other catechols or longer reaction times were used, monoaminated products were only obtained in low yields and undesirable side reactions produced a number of by-products. Reaction kinetics similar to these findings were described for hybrid dimer formation from 3,4-dichloroaniline and syringic acid[24)] or 3-(3,4-dihydroxy-phenyl)-propionic acid and 4-aminobenzoic acid.[25)] In contrast, for hybrid dimer formation from 2,5-dihydroxybenzoic acid derivatives with aminopenicillins and aminocephalosporins,[2,3)] amino acids,[26)] and primary aromatic amines,[27,28)] we found straightforward biotransformations. Therefore the amination of 2,5-dihydroxybenzoic acid derivatives with laccase from *Trametes* sp. is an excellent method for the synthesis of novel β-lactams whereas the amination of catechols is limited to a few specific educts (Fig. 1).

Table 1. Products Obtained in Laccase-Catalyzed Biotransformation

Substances	 **1a**	 **1b**	 **1c**
2a	1[a)] [**3a**][b)] (>5)[c)]	1 [**3e**] (>5)	>5 (>5)
2b	1 [**3b**] (>5)	4 (>5)	>5 (>5)
2c	1 [**3c**] (>5)	4 (>5)	>5 (>5)
2d	1 [**3d**] (>5)	3 (>5)	>5 (>5)

a) Number of products after 1.5 h. *b*) Description of products analyzed in more detail. *c*) Number of products in parentheses after 5 h.

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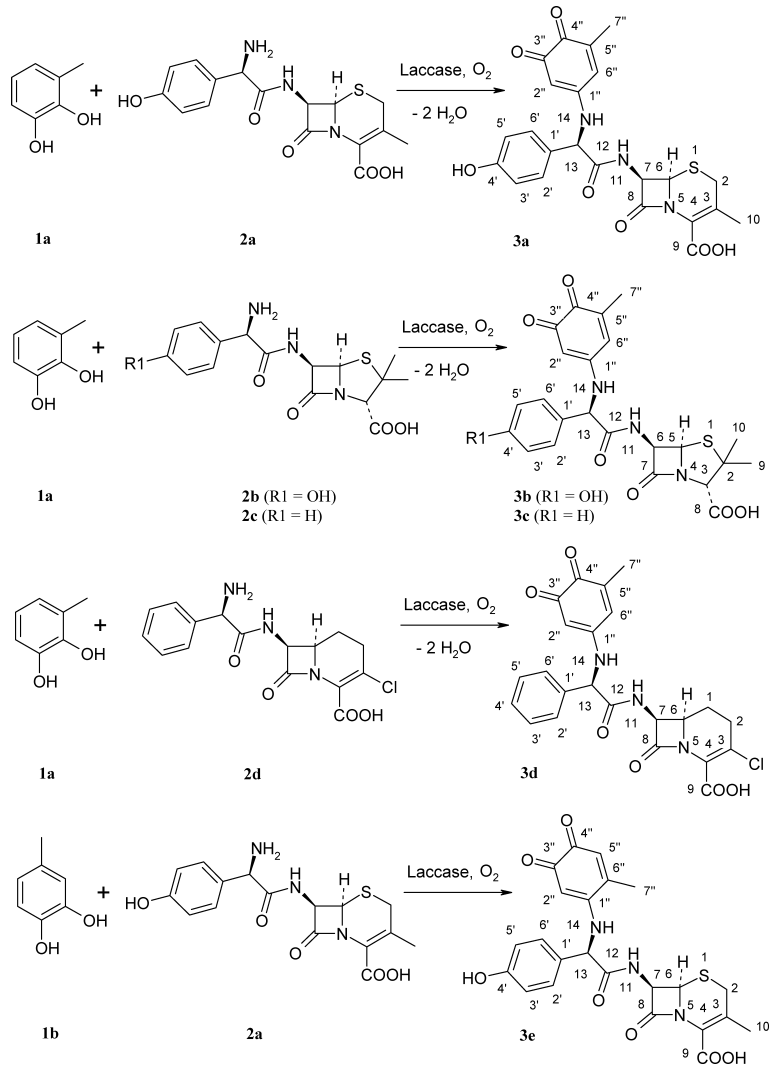

Fig. 1. Catechols (**1a**, **1b**), β-Lactam Antibiotics (**2a** to **2d**), and the Products **3a** to **3e**

After separation of the products **3a** to **3e** by solid phase extraction, mass spectral analyses (LC/MS with API-ES positive modes) of the compounds showed a molecular mass attributed to the coupling of methylcatechol (**1a** or **1b**) with one amino-β-lactam (**2a** to **2d**) under loss of four hydrogen atoms indicating a quinonoid structure of the products. Furthermore, ^{1}H-NMR spectral data of **3a** to **3e** contained the characteristic signals for both substrates (**1a**, **1b**) and educts (**2a** to **2d**). The number of CH proton signals of the dihydroxylated phenyl rings changed from three—in the substrates—to two signals—in the products. The multiplicity of the two signals of the product indicated a further substituent at the C1″. HMBC spectra measured in DMSO showed two typical correlations for quinones in the range of 180 ppm. This is an important indication for the quinonoid character of **3a** to **3e**, confirming the oxidation of catechols to quinones similar to the hybrid dimer quinones formed from 2,5-dihydroxybenzoic acid derivatives with penicillins,[2] amino acids,[26] and primary aromatic amines,[27,28] and for the hybrid dimer quinones produced from 3,4-dichloraniline with protocatechuic acid and syringic acid.[24,29] Although **3a** to **3e** have quinone units as an element of the C-7 or C-6 substituent instead of catechol units these products should also have biological activity because of the described activities of other *ortho*-quinonoid[30—32] or *para*-quinonoid[33—35] substances.

Biological Activity of the Biotransformation Products Growth inhibition of the new β-lactam antibiotics **3a** to **3e** against Gram positive strains was determined by the agar diffusion method according to Burkhardt,[36] and the results for selected strains—among them multidrug resistant *Staphylococcus* and *Enterococcus* strains—are summarized in Table 2. The growth inhibition values of cefadroxil, amoxicillin, ampicillin, and loracarbef (**2a** to **2d**) and the catechol derivatives are also presented for comparison. **3b** and **3c** showed balanced antibacterial activity against all Gram positive strains except against *S. epidermidis* 535 which is strongly resistant to penicillin and cephalosporin families. The new *ortho*-quinonoid substituted penicillins (**3b**, **3c**) showed more potent activity against the tested strains than the new *ortho*-quinonoid substituted cephalosporin and carbacephem derivatives (**3a**, **3d**, **3e**) comparable with the results of the *para*-quinonoid substituted penicillins,[2] cephalosporins, and carbacephems.[3] Investigations towards the stability of the synthesized compounds showed limited lifetime in aqueous solution. Incubation at 30 °C of solutions of **3a** to **3e** showed decomposition after 2 h. Therefore the survey of the antimi-

Table 2. Antimicrobial Activity of Products **3a** to **3e**, and Educts **1a**, **1b**, **2a** to **2d**

Compound	Amount *n* [μmol]	*Entero-coccus faecalis* 769	*Staphylo-coccus aureus* 315	*S. aureus* 36881	*S. aureus* 38418	*S. aureus* 520	*S. aureus* ATCC 6538	*S. aureus* Nord-deutscher Stamm	*S. epider-midis* 125	*S. epider-midis* 535	*S. epider-midis* 847
3a	0.02	r[a)]	r	r	10[b)]	r	16	r	12	r	r
	0.1	r	10	r	18	r	20	10	20	r	14
	0.2	10	14	10	22	10	26	12	26	10	16
3b	0.02	20	r	r	30	r	30	r	20	r	14
	0.1	26	12	12	>30	10	>30	10	24	r	20
	0.2	28	14	14	>30	14	>30	14	28	10	22
3c	0.02	20	r	r	30	10	30	r	20	r	16
	0.1	28	12	12	>30	14	>30	10	26	r	22
	0.2	30	14	14	>30	18	>30	14	28	10	24
3d	0.02	r	r	r	14	r	20	r	14	r	r
	0.1	r	8	r	24	r	30	8	22	r	14
	0.2	r	12	10	28	10	>30	14	26	10	18
3e	0.02	r	r	r	12	r	18	r	16	r	r
	0.1	r	r	r	22	r	30	r	26	r	16
	0.2	r	10	12	26	r	>30	12	28	r	18
2a	0.02	r	r	r	20	r	30	r	22	r	10
	0.1	10	r	14	30	r	>30	r	30	r	22
	0.2	16	r	20	>30	12	>30	r	>30	r	26
2b	0.02	20	r	r	30	r	>30	r	20	r	18
	0.1	26	12	12	>30	10	>30	8	24	r	24
	0.2	28	14	14	>30	14	>30	10	28	r	26
2c	0.02	24	r	r	30	10	30	r	24	r	18
	0.1	28	12	12	>30	14	>30	10	28	r	24
	0.2	30	14	16	>30	18	>30	14	30	10	28
2d	0.02	r	16	r	24	r	28	r	22	r	10
	0.1	r	20	20	30	8	>30	r	30	r	20
	0.2	12	22	28	>30	14	>30	8	>30	r	24
1a	0.02	r	r	r	r	r	r	r	r	r	r
	0.1	r	r	r	r	r	r	r	r	r	r
	0.2	r	r	r	r	r	r	10	8	r	10
1b	0.02	r	r	r	r	r	r	r	r	r	r
	0.1	r	r	r	r	r	r	r	r	r	r
	0.2	r	r	r	r	r	r	10	r	r	r

a) Resistent (no zone of inhibition). *b*) Diameter of inhibition zone (mm).

crobial effects was concentrated on the initial screening using the agar diffusion test.

Cytotoxicity of the new β-lactam antibiotics (**3a** to **3e**) and of cefadroxil, amoxicillin, ampicillin, and loracarbef (**2a** to **2d**) was determined by the neutral red uptake assay[37] using FL-cells, a human amniotic epithelial cell line (Table 3). Whereas **3a** was not cytotoxic against FL cells in concentrations tested, **3b** to **3e** showed slight cytotoxicity. Remarkable is the fact, that the methylcatechol substrates were strong cytotoxic in all concentrations tested whereas cytotoxicity of products (**3b** to **3e**) were comparable low, but not negligible. Aminopenicillins and aminocephalosporins synthesized by amination of 2,5-dihydroxybenzoic acid derivatives showed no or only weak cytotoxicity against FL cells in concentrations up to 100 μg/ml.[2,3]

A "*Staphylococcus*-infected, immune suppressed mouse"

Table 3. Cytotoxic Activity of Products **3a** to **3e**, and Educts **1a**, **1b**, **2a** to **2d**

Compound	IC_{50} (μM)
3a	>200
3b	250
3c	197
3d	142
3e	128
2a	>275
2b	>275
2c	>285
2d	>285
1a	>100
1b	>100

Table 4. Effectiveness of *in Vitro* Active Products in the "*Staphylococcus*-Infected, Immune Suppressed Mouse" Model

Compound	Dose	Survived/treated mice *n*/*n*	Survived/control mice *n*/*n*
	Staphylococcus aureus ATCC 6538		
2a	2×0.5 mg (25 mg/kg)	10/10	0/10
2b	2×0.5 mg (25 mg/kg)	10/10	0/10
3a	2×0.5 mg (25 mg/kg)	3/6	0/10
	2×1.0 mg (50 mg/kg)	1/3	0/5
3b	2×0.5 mg (25 mg/kg)	4/6	0/10
	2×1.0 mg (50 mg/kg)	3/3	0/5
	2×1.5 mg (75 mg/kg)	1/3	0/5
3c	2×0.5 mg (25 mg/kg)	4/6	0/10
	2×1.0 mg (50 mg/kg)	2/3	0/5

model was used for the examination of *in vivo* effectiveness against Gram positive strains of *in vitro* active products (Table 4). 30 to 60% of the mice treated i.p. with biotransformation products survived the infection with *Staphylococcus aureus* ATCC 6538, whereas all untreated mice died after infection within 2 d. But all mice treated with cefadroxil and amoxicillin survived the infection with *Staphylococcus aureus* ATCC 6538 without any noticeable problems, whereas the mice treated with biotransformation products showed symptoms of intoxication. In contrast to these results we reported on *para*-quinonoid substituted penicillins,[2] cephalosporins and carbacephems,[3] which protected mice against an infection with *Staphylococcus aureus* without any hints of intoxication. Of the two series of cephalosporins and penicillins, the ones bearing *para*-quinonoid structures[2,3] showed more potent activity in protecting mice against an infection with *Staphylococcus aureus* than another with *ortho*-quinonoid structures (**3a** to **3e**), possibly due to the different cytotoxicity.

Concluding, the activity *in vivo* of the novel penicillins and cephalosporins with *ortho*-quinonoid structure is unsatisfactory because of the cytotoxicity based on the *ortho*-quinonoid structure. But the laccase-catalyzed amination of catechols with aminopenicillins and aminocephalosporins is a new method to synthesize novel penicillins and cephalosporins by enzymatic transformation. These reactions represent new, low-cost processes, which allow the use of mild reaction conditions, aqueous solvent systems, normal pressure, and room temperature. Further advantage is the specificity of the reaction, which can comprise the amination of *para*- and *ortho*-dihydroxylated aromatic compounds[2,3,25,27,28] on one hand and the combination of two antibiotics containing a phenol moiety,[38] the introduction of a phenolic compound into an antibiotic containing a phenolic moiety,[39] and the oxidation of an antibiotic[40] on the other hand. In summary it can be concluded that the laccase has a high potential for the synthesis of new antibiotic substances. Further studies are in progress.

Experimental

Enzymes Extracellular laccase C of *Trametes* sp. (EC 1.10.3.2) was obtained from ASA Spezialenzyme (Wolfenbüttel, Germany) and used in an activity of 800 nmol·ml^{-1}·min^{-1} (substrate: 2,2′-amino-bis-3-ethylbenzthiazoline-6-sulfonic acid).

Chemicals Chemicals were purchased from commercial suppliers: cefadroxil, cefaclor and cefalexin from Sigma-Aldrich Germany, loracarbef from Eli Lilly Germany, 2,5-dihydroxy-*N*-(2-hydroxyethyl)benzamide from Midori Kagaku Co., Japan, 2,5-dihydroxybenzoic acid methyl ester and 2,5-dihydroxybenzoic acid ethyl ester from Sigma-Aldrich. All chemicals were used as received. 2,5-Dihydroxybenzamide was synthesized as described previously.[28]

Conditions of Biotransformation β-Lactam antibiotics [**2a** to **2d** (13 mg)] were dissolved in 15 ml sodium acetate buffer, 20 mM pH 5.6. After addition of laccase C (activity 800 nmol·ml^{-1}·min^{-1}), the reaction mixtures were completed by a solution of 5 mg catechols (**1a** to **1c**) in 5 ml sodium acetate buffer, pH 5.6. The reaction mixtures were incubated for 5 h at room temperature with agitation at 400 rpm.

For preparative scale the same procedure was used as described above apart from lager amount of educts [**1a** (31 mg in 25 ml for production of **3a**, 9.9 mg in 10 ml for production of **3b**, 37 mg in 25 ml for production of **3c**, 44 mg in 25 ml for production of **3d**), **1b** (31 mg in 25 ml for production of **3e**), **2a**, **2d** (58 mg in 75 ml), **2b** (26 mg in 30 ml), **2c** (49 mg in 75 ml)] and shorter reaction time (1.5 h).

Analytical High-Performance Liquid Chromatography (HPLC) For routine analysis, samples of the incubation mixture were analyzed by an HPLC system (Dionex, Idstein, Germany) consisting of a Gynkotek High Precision Pump Model 480, Dionex ASI-100 Automated Sample-Injector, Dionex UVD 340S Diode-Array-Detector, and Chromeleon Version 6.30. An endcapped, 5-μm, LiChroCart 125-4 RP 18 column (Merck, Darmstadt, Germany) was used. A solvent system consisting of methanol (eluent A) and phosphoric acid, 0.1% pH 2 (eluent B), starting from an initial ratio of 10% A and 90% B and reaching 100% A within 14 min, was used at a flow rate of 1 ml/min.

Procedure for Isolation of Biotransformation Products After activation and equilibration, an RP18 silicagel column (20 cc, 5 g absorbent material, Waters, Manchester, U.K.) was charged with the incubation mixture. The column was washed twice with 20 ml of a mixture of methanol (10%) and distilled water (90%). The products were eluted with 10 ml acetonitrile. The acetonitrile eluates were dried using a vacuum rotator at 30 °C.

Characterization of Biotransformation Products Products were analyzed by mass spectrometry (LC/MS with API-ES in positive modes).

The nuclear magnetic resonance (NMR) spectra were obtained at 600 MHz (^{1}H and HMBC) in dimethyl sulfoxide-d_6 (DMSO-d_6) or acetonitrile-d_3.

7-[2-(5-Methyl-3,4-dioxocyclohexa-1,5-dienylamino)-2-(4-hydroxyphenyl)-acetylamino]-cephalosporanic Acid 3a Synthesis and isolation as described above. Dark red solid. Yield 27% (21 mg). ^{1}H-NMR δ (DMSO-d_6) 1.86 (s, 3H, H7″), 1.99 (s, 3H, H10), 3.29 (d, J=18.4 Hz, 1H, H2), 3.49 (d, J=18.4 Hz, 1H, H2), 4.89 (d, J=4.4 Hz,1H, H6), 5.19 (d(s), J=1.5 Hz, 1H, H2″), 5.31 (d, J=7.0 Hz, 1H, H13), 5.65 (dd, J=4.7, 8.1 Hz, 1H, H7), 6.76 (d, J=8.2 Hz, 2H, H3′, H5′), 7.14 (d(s), J=1.5 Hz, 1H, H6″), 7.27 (d, J=8.2 Hz, 2H, H2′, H6′), 8.71 (d, J=7.1 Hz, 1H, H14), 9.29 (d, J=8.3 Hz, 1H, H11). ^{13}C-NMR δ (DMSO-d_6, determination of carbon assignment by HMBC) 15.6 (C7″), 19.7 (C10), 29.1 (C2), 57.1 (C6), 58.8 (C7), 59.5 (C13), 95.1 (C2″), 115.7 (C3′, C5′), 122.9 (C4), 126.8 (C1′), 128.9 (C2′, C6′), 129.6 (C3), 133.7 (C6″), 140.0 (C5″), 155.2 (C1″), 157.8 (C4′), 163.8 (C9), 164.1 (C8), 170.0 (C12), 174.7 (C3″), 183.6 (C4″). HMBC ^{1}H–^{13}C correlations H7″ (C1″, C4″, C5″, C6″), H10 (C2, C3, C4, C9), H2 (C3, C4, C6, C9, C10), H6 (C2, C8), H2″ (C13, C3″, C4″, C5″, C6″), H13 (C12, C1′, C2′, C6′, C1″), H7 (C6, C8, C12), H3′, H5′ (C1′, C3′, C4′, C5′), H6″ (C2″, C4″, C5″, C7″), H2′,H6′ (C13, C2′, C3′, C4′, C5′, C6′), H14 (C12, C13, C1′, C1″, C2″, C3″, C6″), H11 (C7, C8, C12). LC/MS m/z 483.4 ([M]$^+$, 504.8 [M+Na]$^+$ API-ES pos. mode).

6-[2-(5-Methyl-3,4-dioxocyclohexa-1,5-dienylamino)-2-(4-hydroxyphenyl)-acetylamino]-penicillanic Acid 3b Synthesis and isolation as described above. Dark red solid. Yield 28% (10 mg). ^{1}H-NMR (acetonitrile-d_3) δ 1.46 (s, 3H, H9 or H10), 1.54 (s, 3H, H9 or H10), 4.33 (s, 1H, H3), 5.13 (d, J=6.7 Hz, 1H, H13), 5.14 (s, 1H, H2″), 5.41 (d, J=3.8 Hz,1H, H5), 5.55 (dd, J=3.9, 8.7 Hz, 1H, H6), 6.82 (s, 1H, H6″), 6.84 (d, J=8.4 Hz, 2H, H3′, H5′), 7.29 (d, J=8.3 Hz, 2H, H2′, H6′). LC/MS m/z 485.3 ([M]$^+$, 507.3 [M+Na]$^+$ API-ES pos. mode).

6-[2-(5-Methyl-3,4-dioxocyclohexa-1,5-dienylamino)-2-phenyl-acetylamino]-penicillanic Acid 3c Synthesis and isolation as described above.

Dark red solid. Yield 83% (55 mg). ^{1}H-NMR δ (DMSO-d_6) 1.41 (s, 3H, H9 or H10), 1.55 (s, 3H, H9 or H10), 1.87 (s, 3H, H7″), 4.18 (s, 1H, H3), 5.22 (s, 1H, H2″), 5.39 (d, J=3.7 Hz, 1H, H5), 5.52 (dd, J=3.7, 7.8 Hz, 1H, H6), 5.55 (br s, 1H, H13), 7.18 (s, 1H, H6″), 7.34 (dd, J=7.1 Hz, 1H, H4′), 7.40 (dd, J=7.4, 7.1 Hz, 2H, H3′, H5′), 7.47 (d, J=7.6 Hz, 2H, H2′, H6′), 8.84 (br s, 1H, H14), 9.20 (d, J=7.6 Hz, 1H, H11). ^{13}C-NMR δ (DMSO-d_6, determination of carbon assignment by HMBC) 15.1 (C7″), 26.4 (C9 or C10), 30.1 (C9 or C10), 57.7 (C6), 58.8 (C13), 63.8 (C2), 66.8 (C5), 70.7 (C3), 95.3 (C2″), 127.0 (C2′, C6′), 128.3 (C4′), 128.7 (C3′, C5′), 136.3 (C1′), 133.0 (C6″), 139.8 (C5″), 154.7 (C1″), 168.4 (C12), 169.1 (C8), 172.6 (C7), 182.8 (C4″). HMBC ^{1}H–^{13}C correlations H9, H10 (C3, C5, C9, C10), H7″ (C1″, C4″, C5″, C6″), H3 (C2, C5, C7, C8, C9, C10), H2″ (C3″, C4″, C6″), H5 (C6, C7), H6 (C5, C7, C12), H13 (C12, C1′, C2′, C6′, C1″), H6″ (C2″, C4″, C7″), H4′ (C2′, C6′), H3′, H5′ (C1′, C3′, C5′), H2′, H6′ (C13, C2′, C4′, C6′), H11 (C6, C7, C12). LC/MS m/z 469.4 ([M]$^+$, 491.3 [M+Na]$^+$ API-ES pos. mode).

3-Chloro-7-[2-(5-methyl-3,4-dioxocyclohexa-1,5-dienylamino)-2-phenyl-acetylamino}-8-oxo-5-azabicyclo[4.2.0]oct-3-ene-4-carboxylic Acid 3d Synthesis and isolation as described above. Dark red solid. Yield 81% (63 mg). ^{1}H-NMR δ (DMSO-d_6) 1.35 (m, 2H, H1), 1.86 (s, 3H, H7″), 2.48 (m, 2H, H2), 3.72 (m, J=7.8 Hz, 1H, H6), 5.16 (s, 1H, H2″), 5.25 (dd, J=7.9, 5.6 Hz, 1H, H7), 5.33 (s, 1H, H13), 7.19 (s, 1H, H6″), 7.35 (dd, J=7.4 Hz, 1H, H4′), 7.40 (dd, J=7.4 Hz, 2H, H3′, H5′), 7.49 (d, J=7.6 Hz, 2H, H2′, H6′), 8.88 (br, 1H, H14), 9.39 (d, J=8.0 Hz, 1H, H11). ^{13}C-NMR δ (DMSO-d_6, determination of carbon assignment by HMBC) 21.5 (C1), 28.0 (C2), 51.4 (C6), 57.7 (C7), 60.1 (C13), 95.0 (C2″), 120.2 (C4), 127.2 (C4′), 127.6 (C2′, C6′), 128.5 (C3), 128.7 (C3′, C5′), 133.5 (C6″), 136.5 (C1′), 163.9 (C8), 169.1 (C12), 183.0 (C4″). HMBC ^{1}H–^{13}C correlations H2 (C1, C3, C4, C6), H6 (C2, C8), H2″ (C4″, C6″), H7 (C6, C8, C12), H13 (C12, C1′, C2′, C6′, C1″), H6″ (C2″, C4″, C7″), H4′ (C2′, C6′), H3′,H5′ (C1′, C3′, C5′), H2′, H6′ (C13, C2′, C4′, C6′), H11 (C7, C8, C12). LC/MS m/z 469.5 ([M]$^+$, 491.3 [M+Na]$^+$ API-ES pos. mode).

7-[2-(6-Methyl-3,4-dioxocyclohexa-1,5-dienylamino)-2-(4-hydroxy-phenyl)-acetylamino]-cephalosporanic Acid 3e Synthesis and isolation as described above. Dark red solid. Yield 78% (61 mg). ^{1}H-NMR δ (DMSO-d_6) 1.99 (s, 3H, H10), 2.32 (s, 3H, H7″), 3.29 (d, J=18.1 Hz, 1H, H2), 3.48 (d, J=18.4 Hz, 1H, H2), 4.99 (d, J=4.4 Hz, 1H, H6), 5.12 (s, 1H, H2″), 5.25 (d, J=6.1 Hz, 1H, H13), 5.69 (dd, J=4.9, 7.8 Hz, 1H, H7), 6.32 (s, 1H, H5″), 6.76 (d, J=8.2 Hz, 2H, H3′, H5′), 7.11 (d, J=6.1 Hz, 1H, H14), 7.34 (d, J=8.2 Hz, 2H, H2′, H6′), 9.29 (d, J=8.4 Hz, 1H, H11). ^{13}C-NMR δ (DMSO-d_6, determination of carbon assignment by HMBC) 17.5 (C7″), 19.5 (C10), 28.6 (C2), 56.8 (C6), 58.8 (C7), 59.5 (C13), 97.7 (C2″), 115.9 (C3′, C5′), 123.0 (C4), 126.7 (C1′), 128.7 (C2′, C6′), 129.4 (C5″), 129.6 (C3), 147.2 (C6″), 154.3 (C1″), 158.0 (C4′), 163.6 (C9), 163.9 (C8), 170.2 (C12), 175.6 (C3″), 182.8 (C4″). HMBC ^{1}H–^{13}C correlations H2 (C3, C4, C6, C9, C10), H6 (C2, C8), H2″ (C3″, C4″, C5″, C6″, C7″), H13 (C12, C1′, C2′, C6′, C1″), H7 (C6, C8, C12), H5″ (C1″, C3″, C7″), H3′, H5′ (C1′, C3′, C4′, C5′), H14 (C12, C2″, C6″), H2′, H6′ (C13, C2′, C3′, C4′, C5′, C6′), H11 (C7, C8, C12). LC/MS m/z 483.4 ([M]$^+$, 504.9 [M+Na]$^+$ API-ES pos. mode).

Determination of Antibacterial Activity An agar diffusion method according to Burkhardt[36] was used to determine antibacterial activity. Sterile Mueller-Hinton II-Agar in Stacker petri dishes (Becton Dickinson Microbiology systems, Cockeysville, U.S.A.) was inoculated with bacterial cells (200 μl of bacterial cell suspension—1.5×10^8 cells—on 20 ml medium). The following bacterial strains were used: *Staphylococcus aureus* ATCC 6538 and *S. aureus* Norddeutscher Epidemiestamm. Besides these the agents were tested against the following multidrug resistant strains isolated from patients: *S. aureus* 315, *S. aureus* 36881, *S. aureus* 38418, *S. aureus* 520, *S. epidermidis* 125, *S. epidermidis* 535, *S. epidermidis* 847, and *Enterococcus faecalis* 769. The test samples were applied in different concentrations on sterile paper discs (Sensi-Disc, 6 mm diameter, Becton Dickinson Microbiology systems). Test concentrations were selected according to the concentration of the standard antibiotics (cefadroxil, amoxicillin, ampicillin, and loracarbef) on the Sensi discs. Plates were kept for 3 h in a refrigerator to enable prediffusion of the substances into the agar and were then incubated for 24 h at 37 °C. Average inhibition zone diameters were calculated from 3 replicates.

Cytotoxic Activity The cytotoxicity was determined by the neutral red uptake assay[37] using FL-cells, a human amniotic epithelial cell line. The test allows the differentiation between dead and living cells on the fact that only living cells are able to manage the active uptake of neutral red. FL-cells were cultivated in a 96-well microtiter plate (10^5 cells/ml Hepes modified Dulbecco, Sigma, 150 μl/well) at 37 °C in a humidified 5% carbon dioxide atmosphere. The Dulbecco's Modified Eagle's Medium (DMEM) was completed by L-glutamin (1%, Sigma), penicillin G/streptomycin (1%, Sigma) and FCS (10%, Biocrom). After 24 h 50 μl of the test solution (test substance dissolved in 20 μl DMSO under stirring in an ultrasonic bath for 5 min and then diluted with 1 ml medium) or medium with equal amounts of DMSO (control) were added. After a further incubation for 72 h cells were washed three times with phosphate buffered saline solution (PBS). One hundred microliter neutral red solution (Sigma, 0.3% in DMEM) was added per well. The cells were then incubated for 3 h at 37 °C, followed by another three times washing with PBS. A solution (100 μl) of acetic acid (1%, v/v) and ethanol (50%, v/v) in distilled water were added. After shaking for 15 min the optical density was measured at 492 nm with a Micro Screener LB 9260 (EG&G Berthold, Bad Wildbad, Germany). The mean of three measurements for each concentration was determined (n=3).

Animal Assays A "*Staphylococcus*-infected, immune suppressed mouse" model was established for the examination of *in vivo* effectiveness of *in vitro* selected drugs. In this model 8-weeks old female BALB/C mice (3 mice/group/assay) were pre-treated with cyclophosphamide (250 mg/kg intraperitoneal (i.p.) day −3 and 100 mg/kg i.p. day −1, Sigma) to suppress the immune answer. Three days later they were infected with *Staphylococcus aureus* ATCC 6538, i.p., in a lethal dose (10^{10}—10^{12} colony forming units). The test agents were injected 30 min and 6 h after the infection with *Staphylococcus aureus* ATCC 6538. The concentration of the test agents was selected according to therapeutically used doses of ampicillin. The antibiotic effectiveness was recognised within the next 2—6 d.

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