Novel Cephalosporins Synthesized by Amination of 2,5-Dihydroxybenzoic Acid Derivatives Using Fungal Laccases II[1)]

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Received October 13, 2006; accepted November 24, 2006

Sixteen novel cephalosporins were synthesized by amination of 2,5-dihydroxybenzoic acid derivatives with the aminocephalosporins cefadroxil, cefalexin, cefaclor, and the structurally related carbacephem loracarbef using laccases from *Trametes* sp. or *Myceliophthora thermophila*. All products inhibited the growth of several Gram positive bacterial strains in the agar diffusion assay, among them methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci. The products protected mice against an infection with *Staphylococcus aureus* lethal to the control animals. Cytotoxicity and acute toxicity of the new compounds were negligible. The results show the usefulness of laccase for the synthesis of potential new antibiotics. The biological activity of the new compounds stimulates intensified pharmacological tests.

Key words cephalosporin; laccase; carbacephem; biotransformation; resistance

The laccase-catalyzed amination is a new method to synthesize novel antibiotics by enzymatic transformation.[1)] 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—4)] the combination of two antibiotics containing a phenol moiety,[5)] the introduction of a phenolic compound into an antibiotic containing a phenolic moiety,[6)] the oxidation of an antibiotic,[7)] and the transformation of substituted heterocyclic compounds.[8,9)]

Recently we reported about the derivatization of aminopenicillins coupling them with derivatives of 2,5-dihydroxybenzoic acid.[1)] The motive for our work was the global problem with resistance of many bacteria, *e.g. Streptococcus pneumoniae* strains[10—13)] and *Staphylococcus* strains, against currently available β-lactam antibiotics.[14—17)] The aminated products[1)] inhibited the growth of several Gram positive bacterial strains and protected mice against an infection with *Staphylococcus aureus*.

In this study, we used cephalosporins including the carbacephem loracarbef for laccase-catalyzed reactions because of their advantages over the penicillins. First, they are inherently more stable to β-lactamases. The second advantage is the possible modification of two sites of the cephalosporin molecule, while the penicillin molecule can be modified at only one site. Thus, compared with penicillins, the potential number of cephalosporins is considerably greater. Now we have employed laccases from *Trametes* sp. and from *Myceliophthora thermophila* to achieve derivatization of aminocephalosporins from the first und second broad-spectrum generations and to couple them with derivatives of 2,5-dihydroxybenzoic acid. These derivatives are structurally related to the ganomycins, a new chemical class of antibacterial compounds[18)] and to other antibacterial active isolates,[19,20)] and are therefore interesting as coupling partner for β-lactams. The novel cephalosporins 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 products protected mice against an infection with *Staphylococcus aureus* lethal to the control animals. Cytotoxicity and acute toxicity of the new compounds were negligible.

Results and Discussion

Biotransformation of Aminocephalosporins by Laccases Laccase-catalyzed reaction of 2,5-dihydroxybenzoic acid derivatives (substrates **1a** to **1d**) with cefadroxil, cefalexin, cefaclor, and loracarbef (educts **2a**—**d**) proceeded very fast. Using the laccase from *Trametes* sp., the compounds were consumed after an incubation period of 3 h resulting in any case in one cross coupling product (**3a** to **3p**, Fig. 1).

Comparable straight forward biotransformation of educts to products we found also for hybrid dimer formation from 2,5-dihydroxybenzoic acid derivatives with aminopenicillins,[1)] amino acids,[21)] and primary aromatic amines,[2,3)] and from 3-(3,4-dihydroxy-phenyl)-propionic acid with 1-hexylamine.[4)] Different reaction kinetics were described for hybrid dimer formation from 3,4-dichloroaniline and syringic acid[22)] or 3-(3,4-dihydroxy-phenyl)propionic acid and 4-aminobenzoic acid.[4)] Therefore the amination of 2,5-dihydroxybenzoic acid derivatives with laccase from *Trametes* sp. is an excellent method for the synthesis of novel cephalosporins. In contrast when using laccase from *Myceliophthora thermophila*, diaminated products and some not identified byproducts were formed as reported for amination with primary aromatic amines.[3)] These undesirable reactions diminished product yields up to 50%.

After separation of the products **3a** to **3p** by solid phase extraction, mass spectral analyses (LC/MS with API-ES in negative and positive modes) of the compounds showed a molecular mass attributed to the coupling of one 2,5-

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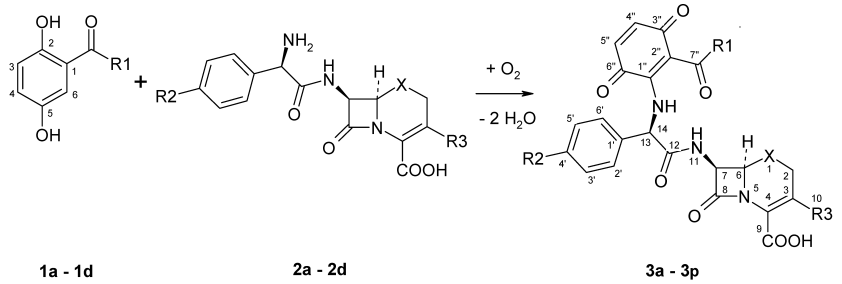

	R1
1a	$NH(CH_2)_2OH$
1b	NH_2
1c	OCH_3
1d	OCH_2CH_3

	R2	R3	X
2a	OH	CH_3	S
2b	H	CH_3	S
2c	H	Cl	S
2d	H	Cl	CH_2

	R1	R2	R3	X
3a		OH	CH_3	S
3e	8′′ 9′′ 10′′	H	CH_3	S
3i	$NHCH_2CH_2OH$	H	Cl	S
3m		H	Cl	CH_2
3b		OH	CH_3	S
3f	8′′	H	CH_3	S
3j	NH_2	H	Cl	S
3n		H	Cl	CH_2
3c		OH	CH_3	S
3g	8′′	H	CH_3	S
3k	OCH_3	H	Cl	S
3o		H	Cl	CH_2
3d		OH	CH_3	S
3h	8′′ 9′′	H	CH_3	S
3l	OCH_2CH_3	H	Cl	S
3p		H	Cl	CH_2

Fig. 1. 2,5-Dihydroxybenzoic Acid Derivatives (**1a** to **1d**), β-Lactam Antibiotics (**2a** to **2d**) and the Products **3a** to **3p**

dihydroxybenzoic acid derivative (**1a** to **1d**) with one cephalosporin derivative (**2a** to **2d**) under loss of four hydrogen atoms. These couplings were confirmed by the presence of all carbons of the 2,5-dihydroxy-*N*-(2-hydroxyethyl)benzamide **1a** and of cefadroxil **2a**, cefalexin **2b**, cefaclor **2c**, or loracarbef **2d** in the ^{13}C-NMR spectra of the products **3a**, **3e**, **3h**, and **3l**. Two signals in the range of 180 ppm indicated a quinonoid character of the products. Furthermore, ^{1}H-NMR spectral data of all products showed characteristic signals for both compounds. The number of CH proton signals of the dihydroxylated phenyl rings changed from three—in the educts—to two signals—in the products. The multiplicity of the signals of the C-4″ and C-5″ protons indicated a further substituent at the C-1″ position. The chemical shift to lower field of the C-4″ and C-5″ protons demonstrated the presence of an electron-withdrawing group. Signals for phenolic hydroxy groups could not be detected, but signals for three amine protons were observed. All results together confirm the oxidation of the *p*-hydroquinone to a quinone as described previously for hybrid dimer formation from 2,5-dihydroxybenzoic acid derivatives with penicillins,[1)] amino acids,[21)] and primary aromatic amines,[2,3)] and for the hybrid dimer formation from 3,4-dichloraniline with protocatechuic acid and syringic acid.[22,23)]

Biological Activity of the Biotransformation Products

All products (**3a** to **3p**) showed a moderate to strong growth inhibition of several Gram positive strains, among them multidrug resistant *Staphylococcus* and *Enterococcus* strains, and of the Gram negative strain *E. coli* in the agar diffusion assay (Table 1, data for **3a** to **3d,** all other data in supporting information available *via* e-mail: annett.mikolasch@gmx.de), but not against *Pseudomonas aeruginosa* and *P. maltophilia*. Investigations towards the stability of the products showed limited lifetime in aqueous solution. Incubation of solutions of **3a** to **3p** at 30 °C showed decomposition after 2 h. Therefore the survey of the antimicrobial effects were concentrated on the initial screening using the agar diffusion test. **3a** to **3p** showed no or only weak cytotoxicity against FL cells in concentrations up to 100 μg/ml (data not shown). A "*Staphylococcus*-infected, immune suppressed mouse" model was used for the examination of *in vivo* effectiveness of *in vitro* active products (Table 2, data for **3a**, **3e**, **3i**, **3m**, all other data in supporting information available *via* e-mail: annett.mikolasch@gmx.de).

The mice treated i.p. with one of the biotransformation products survived the infection with *Staphylococcus aureus* ATCC 6538 whereas untreated mice died after infection within 2 d. The treated mice did not show any signs of intoxication. Besides this, **3a** was tested in higher concentration (2500 mg/kg i.p., observation period 20 d after injection) or repeated application (50 mg/kg i.p. two times per day for 14 d, observation period 30 d starting with the first day of injection) for possible toxic effects in mice. Both schedules did not show any toxic side effect during the observation period. These results showed that, as is the case for aminopenicillins,[1)] derivatization of cephalosporins by laccase-catalyzed reaction can be done without a loss of antibacterial activity, whereas the phenolic oxidation of 7-(4-hydroxyphenylacetamido)cephalosporinic acid[7)] and the dimerization of penicillin X[24)] by laccase led to a distinct loss of antibacterial activity.

Corresponding to the aminopenicillin derivatives resulting from laccase-catalyzed amination,[1)] the new cephalosporin derivatives did not show an increase in activity compared to the β-lactam educts. No considerable differences between the coupling products or between coupling products and

Table 1. Antimicrobial Activity of Products **3a** to **3d** and **2a**

Strain	Amount n (μmol)														
	2a			**3a**			**3b**			**3c**			**3d**		
	0.019	0.1	0.19	0.019	0.1	0.19	0.019	0.1	0.19	0.019	0.1	0.19	0.029	0.14	0.29
Bacillus megaterium SBUG 1152	36[a)]	38	40	22	30	32	22	30	32	22	30	32	18	30	34
B. subtils AWD 166	36	38	40	24	30	32	22	30	32	22	30	32	22	30	34
Escherichia coli SBUG 1135	18	22	26	r[b)]	14	16	r	14	18	r	14	18	r	12	16
Enterococcus faecalis 769	r	10	14	r	r	r	r	r	r	r	r	r	r	r	r
E. faecalis 945	r	r	r	r	r	r	r	r	r	r	r	r	r	r	r
Staphylococcus aureus 315	r	r	8	r	10	16	r	r	8	r	r	12	r	r	8
S. aureus 33490	16	24	28	r	14	18	r	14	20	r	14	20	r	14	16
S. aureus 34289	r	r	r	r	r	r	r	r	12	r	r	r	r	r	r
S. aureus 36881	r	14	18	r	10	16	r	12	18	r	r	14	r	14	18
S. aureus 38418	30	34	38	14	24	26	16	24	26	10	22	28	10	20	24
S. aureus 39105	16	24	26	r	r	14	r	12	18	r	r	12	r	r	14
S. aureus 520	r	r	8	r	r	12	r	r	r	r	r	r	r	r	r
S. aureus 526	r	r	r	r	r	10	r	r	12	r	r	r	r	r	r
S. aureus ATCC 6538	32	38	40	20	28	30	16	24	28	18	26	30	14	22	26
S. aureus Nordeutscher Stamm	r	r	10	r	16	18	r	8	12	r	10	14	r	10	12
S. epidermidis 1068	20	24	26	r	10	16	r	16	20	r	14	20	r	12	14
S. epidermidis 1071	22	28	30	r	18	20	r	18	22	r	14	20	r	14	18
S. epidermidis 125	26	36	40	14	24	28	14	22	26	12	24	28	10	20	24
S. epidermidis 563	24	28	32	r	10	16	r	14	20	r	14	20	r	12	18
S. epidermidis 847	12	26	30	r	16	20	r	10	12	r	14	18	r	10	14

a) Zones of inhibition (mm). *b*) r resistent (no zone of inhibition).

Table 2. Effectiveness of *in Vitro* Active Products in the "*Staphylococcus*-Infected, Immune Suppressed Mouse" Model

Product	Concentration	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
3a	2×0.5 mg (25 mg/kg)	9/9	0/15
3e	2×0.5 mg (25 mg/kg)	3/3	0/5
3i	2×0.5 mg (25 mg/kg)	12/12	0/20
3m	2×0.5 mg (25 mg/kg)	9/9	0/15

aminocephalosporins concerning spectrum and strength of antibacterial activity were found until now. However, derivatization can also influence other features of the antibacterial compounds favorable. Thus, we reported about a coupling product, which was quite stable against different types of β-lactamases whereas aminopenicillins were reduced in their activity against *Staphylococcus aureus* SG10 after treatment with β-lactamases.[1)] Furthermore, the positive log D values of the coupling products in contrast to the negative log D of amoxicillin and ampicillin suggest that the compounds could be adsorbed from the gastrointestinal tract after peroral application.[1)] Studies regarding the stability against β-lactamases, the log D values, acute and chronic toxicity, activity after peroral application and pharmacokinetic behaviour as well as investigations towards the enhancement of stability for the novel cephalosporins are in progress.

Concluding, the laccase-catalyzed amination of 2,5-dihydroxybenzoic acid derivatives with aminocephalosporins is a new method to synthesize novel cephalosporins by enzymatic transformation. The novel cephalosporins broaden the spectrum of β-lactam antibiotics. The transfer of the laccase-catalyzed synthesis on cephalosporins and penicillins[1)] shows the enormous potential of this method for the synthesis of new bioactive compounds.

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).

Laccase from *Myceliophthora thermophila* (expressed in genetically modified *Aspergillus* sp.) was bought from NovoNordisk (Bagsvaerd, Denmark). It was used in an activity of 800 nmol·ml^{-1}·min^{-1} (substrate: 2,2′-amino-bis-3-ethylbenzthiazoline-6-sulfonic acid).

Substrates and Conditions of Biotransformation Aminocephalosporins—cefadroxil, cefalexin, cefaclor, and carbacephem loracarbef (2 mM)—were dissolved in 600 ml sodium acetate buffer, 20 mM pH 5.6. After addition of laccase C (activity 800 nmol·ml^{-1}·min^{-1}), the reaction mixture was completed by a 20 mM solution of one of the following 2,5-dihydroxybenzoic acid derivatives in 60 ml sodium acetate buffer, pH 5.6: 2,5-dihydroxy-*N*-(2-hydroxyethyl)benzamide, 2,5-dihydroxybenzamide, 2,5-dihydroxybenzoic acid methyl ester or 2,5-dihydroxybenzoic acid ethyl ester. The reaction mixture was incubated for 3 h at room temperature (RT) with agitation at 400 rpm.

For reaction with laccase from *Myceliophthora thermophila* (activity 800 nmol·ml^{-1}·min^{-1}), the aminocephalosporins were incubated in citrate-phosphate buffer (16 mM citrate, 164 mM phosphate) pH 7. The 2,5-dihydroxybenzoic acid derivatives were solved in 60 ml citrate-phosphate buffer. Further reaction steps were done as described for transformation with laccase C.

Chemicals were purchased from commercial suppliers: cefadroxil, cefaclor and cefalexin from Sigma-Aldrich, loracarbef from Eli Lilly, 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.[3)]

Analytical High-Performance Liquid Chromatography (HPLC) and General Procedure for Isolation of Biotransformation Products For routine analysis, samples of the incubation mixture were analyzed by HPLC

as described prevously.[1)] After 3 h the two educts of each reaction were transformed by laccase from *Trametes* sp. in any case to one product. All isolation steps of the coupling products were performed by solid phase extraction corresponding to the method for transformed aminopenicillins.[1)]

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

The nuclear magnetic resonance (NMR) spectra were obtained at 300 MHz (^{1}H) and at 75 MHz (^{13}C and DEPT-135) in acetonitrile-d_3.

6-{2-[2-(2-Hydroxyethylcarbamoyl)-3,6-dioxocyclohexa-1,4-dienylamino]-2-(4-hydroxyphenyl)-acetylamino}-cephalosporanic Acid 3a Synthesis and isolation as described above. Dark red solid. Yield 88% (930.0 mg). ^{1}H-NMR δ 2.01 (s, 3H, H-10), 3.19 (d, J=18.3 Hz, 1H, H-2), 3.37 (m, J=5.7 Hz, 2H, H-9″), 3.43 (d, J=18.3 Hz, 1H, H-2), 3.57 (t, J=5.5 Hz, 2H, H-10″), 4.89 (d, J=4.7 Hz, 1H, H-6), 5.65 (dd, J=4.7, 8.9 Hz, 1H, H-7), 5.87 (d, J=6.5 Hz, 1H, H-13), 6.56 (d, J=10.2 Hz, 1H, H-4″), 6.67 (d, J=10.1 Hz, 1H, H-5″), 6.82 (d, J=8.7 Hz, 2H, H-3′, H-5′), 7.39 (d, J=8.7 Hz, 2H, H-2′, H-6′), 7.52 (d, J=8.6 Hz, 1H, H-11), 9.77 (t, 1H, H-8″), 13.12 (d, 1H, J=6.2 Hz, H-14). ^{13}C-NMR δ 19.91 (C-10), 30.34 (C-2), 42.10 (C-9″), 57.97 (C-6), 59.82 (C-7), 61.52 (C-10″), 62.95 (C-13), 101.39 (C-2″), 116.65 (C-3′, C-5′), 123.15 (C-4), 129.83 (C-1′), 129.96 (C-2′, C-6′), 132.41 (C-3), 133.33 (C-4″), 141.34 (C-5″), 153.46 (C-1″), 158.46 (C-4′), 164.01 (C-8), 165.05 (C-9), 170.12 (C7″), 171.96 (C-12), 184.60 (C-6″), 185.52 (C-3″). LC/MS m/z 557.5 ([M+H]$^+$, 597.5 [M+Na]$^+$ API-ES pos. mode).

6-{2-[2-(2-Hydroxyethylcarbamoyl)-3,6-dioxocyclohexa-1,4-dienylamino]-2-phenyl-acetylamino}-cephalosporanic Acid 3e Synthesis and isolation as described above. Dark red solid. Yield 74% (150.5 mg). ^{1}H-NMR δ 2.01 (s, 3H, H-10), 3.15 (d, J=18.4 Hz, 1H, H-2), 3.37 (m, J=5.7 Hz, 2H, H-9″), 3.44 (d, J=18.4 Hz, 1H, H-2), 3.57 (t, J=5.6 Hz, 2H, H-10″), 4.90 (d, J=4.7 Hz, 1H, H-6), 5.69 (dd, J=4.6, 8.9 Hz, 1H, H-7), 6.00 (d, J=6.5 Hz, 1H, H-13), 6.59 (d, J=10.2 Hz, 1H, H-4″), 6.71 (d, J=10.1 Hz, 1H, H-5″), 7.46 (m, 6H, H-2′, H-3′, H-4′, H-5′, H-6′, H-11), 9.79 (t, 1H, H-8″), 13.29 (d, 1H, J=6.2 Hz, H-14). ^{13}C-NMR δ 19.93 (C-10), 30.35 (C-2), 42.10 (C-9″), 57.95 (C-6), 59.81 (C-7), 61.50 (C-10″), 63.15 (C-13), 101.42 (C-2″), 123.15 (C-4), 128.41 (C-2′, C-6′), 129.84 (C-4′), 130.09 (C-3′, C-5′), 132.41 (C-3), 133.33 (C-4″), 138.36 (C-1′), 141.34 (C-5″), 153.39 (C-1″), 161.46 (C-8), 165.01 (C-9), 170.12 (C7″), 171.96 (C-12), 184.60 (C-6″), 185.52 (C-3″). LC/MS m/z 543.1 ([M+H]$^+$, 563.0 [M+Na]$^+$ API-ES pos. mode).

3-Chloro-7-{2-[2-(2-hydroxyethylcarbamoyl)-3,6-dioxocyclohexa-1,4-dienylamino]-2-phenyl-acetylamino}-8-oxo-1-thia-5-azabicyclo[4.2.0]-oct-3-en-4-carboxylic Acid 3i Synthesis and isolation as described above. Dark red solid. Yield 87% (73.7 mg). ^{1}H-NMR δ 3.36 (m, J=5.7 Hz, 2H, H-9″), 3.38 (d, J=18.2 Hz, 1H, H-2), 3.57 (t, J=5.6 Hz, 2H, H-10″), 3.75 (d, J=18.2 Hz, 1H, H-2), 4.99 (d, J=4.9 Hz, 1H, H-6), 5.72 (dd, J=4.8, 9.0 Hz, 1H, H-7), 5.94 (d, J=6.7 Hz, 1H, H-13), 6.56 (d, J=10.1 Hz, 1H, H-4″), 6.71 (d, J=10.1 Hz, 1H, H-5″), 7.42 (m, 5H, H-2′, H-3′, H-4′, H-5′, H-6′), 7.66 (d, J=8.9 Hz, 1H, H-11), 9.76 (t, 1H, H-8″), 13.25 (d, 1H, J=6.2 Hz, H-14). ^{13}C-NMR δ 31.27 (C-2), 42.11 (C-9″), 58.09 (C-6), 60.04 (C-7), 61.48 (C-10″), 63.48 (C-13), 101.40 (C-2″), 124.60 (C-4), 128.41 (C-2′, C-6′), 129.13 (C-3), 129.84 (C-4′), 130.09 (C-3′, C-5′), 133.36 (C-4″), 138.69 (C-1′), 141.89 (C-5″), 153.55 (C-1″), 162.30 (C-8), 165.83 (C-9), 170.10 (C7″), 171.37 (C-12), 184.75 (C-6″), 185.51 (C-3″). LC/MS m/z 562.2 ([M+H]$^+$, 584.1 [M+Na]$^+$ API-ES pos. mode).

3-Chloro-7-{2-[2-(2-hydroxyethylcarbamoyl)-3,6-dioxocyclohexa-1,4-dienylamino]-2-phenyl-acetylamino}-8-oxo-5-azabicyclo[4.2.0]oct-3-ene-4-carboxylic Acid 3m Synthesis and isolation as described above. Dark red solid. Yield 77% (87.5 mg). ^{1}H-NMR δ 1.33 (m, 2H, H-1), 2.40 (m, 2H, H-2), 3.37 (m, J=5.6 Hz, 2H, H-9″), 3.58 (t, J=5.5 Hz, 2H, H-10″), 3.74 (m, J=5.0 Hz, 1H, H-6), 5.31 (m, J=5.0, 8.3 Hz, 1H, H-7), 5.90 (d, J=6.7 Hz, 1H, H-13), 6.55 (d, J=10.2 Hz, 1H, H-4″), 6.66 (d, J=10.2 Hz, 1H, H-5″), 7.41 (m, 5H, H-2′, H-3′, H-4′, H-5′, H-6′), 7.54 (d, J=7.9 Hz, 1H, H-11), 9.76 (t, 1H, H-8″), 13.23 (d, 1H, J=6.3 Hz, H-14). ^{13}C-NMR δ 22.19 (C-1), 31.70 (C-2), 42.12 (C-9″), 53.13 (C-6), 59.24 (C-7), 61.51 (C-10″), 63.62 (C-13), 101.65 (C-2″), 124.74 (C-4), 128.33 (C-2′, C-6′), 129.03 (C-3), 129.79 (C-4′), 130.07 (C-3′, C-5′), 133.34 (C-4″), 138.96 (C-1′), 141.92 (C-5″), 153.47 (C-1″), 162.22 (C-8), 165.73 (C-9), 170.09 (C7″), 171.37 (C-12), 184.69 (C-6″), 185.49 (C-3″). LC/MS m/z 544.2 ([M+H]$^+$, 566.1 [M+Na]$^+$ API-ES pos. mode).

The data of all other biotransformation products are in supporting information available *via* e-mail: annett.mikolasch@gmx.de.

Determination of Antibacterial Activity An agar diffusion method according to BURKHARDT[25)] 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—15×10^7 cells—on 20 ml medium). The following bacterial strains were used: *Bacillus megaterium* SBUG 1152, *Bacillus subtilis* AWD 166, *Staphylococcus aureus* ATCC 6538, *S. aureus* Norddeutscher Epidemiestamm and *Escherichia coli* SBUG 1135. Besides these the agents were tested against the following multidrug resistant strains isolated from patients: *S. aureus* 315, *S. aureus* 33490, *S. aureus* 34289, *S. aureus* 36881, *S. aureus* 38418, *S. aureus* 39105, *S. aureus* 520, *S. aureus* 526, *S. epidermidis* 1068, *S. epidermidis* 1071, *S. epidermidis* 125, *S. epidermidis* 535, *S. epidermidis* 563, *S. epidermidis* 847, *Enterococcus faecalis* 769, *E. faecalis* 945, *Pseudomonas aeruginosa* 396, *P. aeruginosa* 595 and *P. maltophilia* 135. 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, cefalexin, cefaclor, 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[26)] using FL-cells, a human amniotic epithelial cell line. 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). 100 μl 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. 100 μl of a solution 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). 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 intra peritoneal (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 (CFU)). 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 therapeutic used doses of ampicillin. The antibiotic effectiveness was recognised within the next 2—6 d.

Preliminary toxicity assays were done with one coupling product (**3a**) using 8 weeks old female BALB/C mice. The compound was injected at a dose of 2500 mg/kg i.p. Its influence was recognised by daily observation of the mice within the next 20 d. In another set of experiments mice were treated with compound **3a** (50 mg/kg i.p. per injection) two times per day over a period of 14 d.

Acknowledgment We thank M. Kindermann, S. Siegert, B. Witt, and A. Neumann for providing NMR data, and S. Sokolowski for LC/MS data. Part of this work was supported financially by the Stiftung Industrieforschung (S574).

References and Notes

1) "Enzymatic Synthesis and Activity of Novel β-Lactam Antibiotics", for Part I see: Mikolasch A., Niedermeyer T. H. J., Lalk M., Witt S., Seefeldt S., Hammer E., Schauer F., Gesell M., Hessel S., Jülich W. D., Lindequist U., *Chem. Pharm. Bull.*, **54**, 632—638 (2006).
2) Manda K., Hammer E., Mikolasch A., Niedermeyer T. H. J., Dec J., Jones A. D., Benesi A. J., Schauer F., Bollag J.-M., *J. Mol. Catal. B: Enzymatic*, **35**, 86—92 (2005).
3) Niedermeyer T. H. J., Mikolasch A., Lalk M., *J. Org. Chem.*, **70**, 2002—2008 (2005).

4) Mikolasch A., Hammer E., Jonas U., Popowski K., Stielow A., Schauer F., *Tetrahedron*, **58**, 7589—7593 (2002).
5) Agematu H., Tsuchida T., Kominato K., Shibamoto N., Yoshioka T., Nishida H., Okamoto R., Shin T., Murao S., *J. Antibiot.*, **46**, 141—148 (1993).
6) Anyanwutaku I. O., Petroski R. J., Rosazza J. P. N., *Bioorg. Med. Chem.*, **2**, 543—551 (1994).
7) Agematu H., Kominato K., Shibamoto N., Yoshioka T., Nishida H., Okamoto R., Shin T., Murao S., *Biosci. Biotechnol. Biochem.*, **57**, 1387—1388 (1993).
8) Schäfer A., Specht M., Hetzheim A., Francke W., Schauer F., *Tetrahedron*, **57**, 7693—7699 (2001).
9) Mikolasch A., Schauer F., *J. Basic Microbiol.*, **43**, 508—521 (2003).
10) Appelbaum P. C., *Eur. J. Clin. Microbiol.*, **6**, 367—377 (1987).
11) Critchley I. A., Karlowsky J. A., Draghi D. C., Jones M. E., Thornsberry C., Murfitt K., Sahm D. F., *Antimicrob. Agents Chemother.*, **46**, 550—555 (2002).
12) Fluit A. C., Schmitz F. J., Jones M. E., Acar J., Gupta R., Verhoef J., *Int. J. Infect. Dis.*, **3**, 153—156 (1997).
13) Klugman K. P., *Clin. Microbiol. Rev.*, **3**, 171—196 (1990).
14) Shimizu M., Shiota S., Mizushima T., Ito H., Hatano T., Yoshida T., Tsuchiya T., *Antimicrob. Agents Chemother.*, **45**, 3198—3201 (2001).
15) Fisher J. F., Meroueh S. O., Mobashery S., *Chem. Rev.*, **105**, 395—424 (2005).
16) Waley S. G., "The Chemistry of β-Lactams," ed. by Page M. I., Blackie A. & P., London, United Kingdom, 1992, pp. 198—228.
17) Helfand M. S., Bonomo R. A., *Curr. Opin. Pharmacol.*, **5**, 452—458 (2005).
18) Mothana R. A. A., Jansen R., Jülich W. D., Lindequist U., *J. Nat. Prod.*, **63**, 416—418 (2000).
19) Fokialakis N., Magiatis P., Chinou I., Mitaku S., Tillequin F., *Chem. Pharm. Bull.*, **50**, 413—414 (2002).
20) Yaoita Y., Danbara K., Kikuchi M., *Chem. Pharm. Bull.*, **53**, 1202—1203 (2005).
21) Manda K., Hammer E., Mikolasch A., Gördes D., Thurow K., Schauer F., *Amino Acids*, **31**, 409—419 (2006).
22) Tatsumi K., Freyer A., Minard R. D., Bollag J. M., *Environ. Sci. Technol.*, **28**, 210—215 (1994).
23) Tatsumi K., Freyer A., Minard R. D., Bollag J. M., *Soil Biol. Biochem.*, **26**, 735—742 (1994).
24) Agematu H., Tsuchida T., Kominato K., Shibamoto N., Yoshioka T., Nishida H., Okamoto R., Shin T., Murao S., *J. Antibiot.*, **46**, 141—148 (1993).
25) Burkhardt F., "Mikrobiologische Diagnostik," Georg Thieme Verlag, Stuttgart, New York, 1992.
26) Lindl T., Bauer J., "Zell und Gewebekultur," Gustav Fischer Verlag, Jena, 1989.