USE OF AMINO ACID *N*-CARBOXY ANHYDRIDE IN THE SYNTHESIS OF PEPTIDE PRODRUG DERIVATIVES (INCLUDING β-CHLOROALANYL) OF C4-β-AMINOALKYL CARBAPENEMS. *IN VITRO* AND *IN VIVO* ACTIVITIES†

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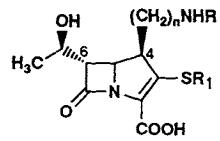
(Received for publication July 27, 1992)

Mono- and dipeptide derivatives of C4-β-aminoalkyl carbapenems were synthesized by the use of amino acid *N*-carboxy anhydride for the peptide bond formation. They were shown to act as prodrugs *in vivo* while imparting the much desired chemical stability. The β-chloroalanyl derivative was suggested to act, in part, as a "dual-purpose" antibacterial.

Since the discovery of thienamycin/imipenem,[1,2] a great deal of attention has been focussed on synthesizing carbapenem antibiotics with different entities around the basic skeleton including those with an aminoethyl moiety at C-6[3,4] and more recently a β-aminoalkyl group at C-4.[5~9] The latter series of compounds (**1**) exhibited extraordinary antibacterial activities, especially against *Pseudomonas aeruginosa*, similar to C4-β-aminoalkoxy compounds.[10]

The 4-aminoethyl derivatives (**1**, n=2, R=H, R_1= a wide variety of substituents), the most active compounds in this series, have been found to possess limited stability at physiological pH (7.4), and becoming more unstable with increasing pH. In fact compound **2** at pH 10 has been shown to rearrange to **3** *via* an intramolecular transacylation.[11]

In this paper we describe acylation of the amine group of **4** and **5** to decrease its basicity/nucleophilicity, and in turn increase their chemical stability. The mono- and dipeptide derivatives are shown to be useful as prodrugs *in vivo*. Also, a β-chloroalanine dipeptide conjugate is conceived



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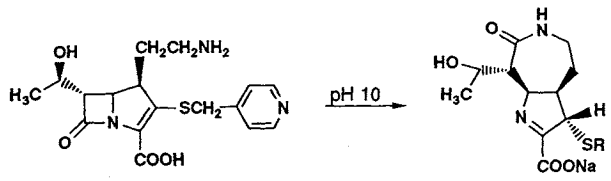


2 **3** R=4-picolyl

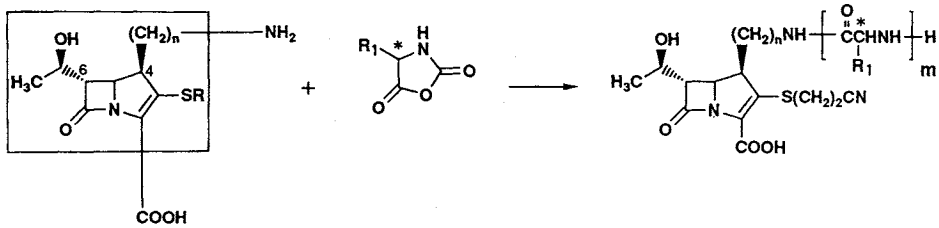
† First disclosed at the "31st Interscience Conference on Antimicrobial Agents and Chemotherapy" held in Chicago, September 29~October 2, 1991, Abstract No. 830.

and shown to act as a "dual-purpose" antibacterial *in vivo*, at least in part.

Chemistry

The peptide derivatives shown in Scheme 1 were prepared employing *N*-carboxyanhydride (NCA) of the corresponding amino acid. The usefulness of NCA as the amino protected, carboxyl activated amino acid for the peptide bond formation with an unprotected amino acid in aqueous medium was recognized quite some time ago[12,13] although it was never applied generally because of 1) its narrow range of experimental conditions and the need for optimization of individual reaction, and 2) side reactions, especially the so called "over reaction" in which tri- and/or oligopeptides were also formed. An attractive feature of this reaction is the lack of racemization. The carbapenems **4** and **5** might be viewed as amino acids with the portion enclosed in the box as the side chain. Their water solubility allowed them to be reacted with the NCAs without the protection of the hydroxyl group in the C-6 side chain. The NCAs were prepared by treatment of the amino acids with trichloromethyl chloroformate in THF at 50°C and precipitating them from ethyl acetate solution with hexane. The *N*-acylations of **4** and **5** with NCAs were carried out in aqueous solution with dioxane as the cosolvent at pH 10.2. Under these conditions both the mono- and dipeptides were formed, the latter as a result of "over reaction", which was used to our advantage to procure enough quantities for biological evaluation, thus avoiding an elaborate synthetic methodology. The ^{1}H NMR spectra of **9** and **10** did not show the presence of **11** and **12**, respectively and *vice versa*, providing evidence that racemization did not occur.[12,13] The dipeptide was also obtained from the monopeptide by reacting it with the NCA under the same reaction conditions. In order to prevent the formation of the "over reaction" product **10**, the reaction was carried out with an alternative acylating species, **17** obtained by the addition of a stoichoiometric amount of DMAP to a solution of **6** in dioxane. Also, using **17** the monopeptide **9** was cleanly converted to the dipeptide **10**. It is noteworthy that the reaction time was relatively longer in the formation of **15** and **16** from **5** and **8**, which may be attributed

Scheme 1.

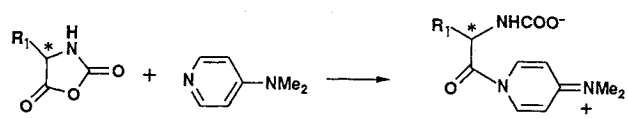


4 n=2, R=$(CH_2)_2CN$
5 n=4, R=$(CH_2)_2CN$

6 $R_1=CH_3$ (*D)
7 $R_1=CH_3$ (*L)
8 $R_1=CH_2Cl$ (*L)

9 n=2, m=1, $R_1=CH_3$ (*D);
11 n=2, m=1, $R_1=CH_3$ (*L);
13 n=2, m=1, $R_1=CH_2Cl$ (*L);
15 n=4, m=1, $R_1=CH_2Cl$ (*L);

10 dipeptide (m=2)
12 dipeptide (m=2)
14 dipeptide (m=2)
16 dipeptide (m=2)



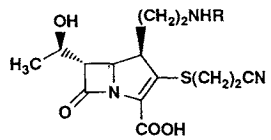
6 **17**

Table 1. *In vitro* (minimum inhibitory concentration, μg/ml)[a] and *in vivo* (PD_{50})[b] data for *N*-acyl derivatives, and their chemical stability ($T_{1/2}$)[c].

Bacterial species	MIC				
	4	**18**	**19**	**20**	Imipenem
Streptococcus pneumoniae (*S. pn*) A9585	0.13	0.06	0.25	0.5	0.001
S. pyogenes (*S. py*) A9604	0.13	0.06	0.13	0.25	0.001
Enterococcus faecalis (*E. fae*) A20688	16	64	32	32	0.5
Staphylococcus aureus (*S. au*) A9537	1	0.5	1	0.5	0.007
S. aureus MR[d] (*S. au* MR) A20699	32	>128	2	0.5	32
Escherichia coli (*E. coli*) A15119	0.008	0.03	0.13	0.13	0.125
Klebsiella pneumoniae (*K. pn*) A9664	0.03	0.06	0.03	0.13	0.125
Enterobacter cloacae (*E. clo*) A9659	0.25	4	0.5	2	0.5
Proteus mirabilis (*P. mir*) A9900	1	—	0.5	1	1
P. vulgaris (*P. vul*) A21559	0.25	0.25	0.5	0.06	1
Providencia rettgeri (*P. rett*) A22424	2	1	0.5	2	1
Pseudomonas aeruginosa (*Ps. ae*) A9843	0.25	128	2	32	1
	PD_{50}				
Pseudomonas aeruginosa (*Ps. ae*) A9843	0.2	>25	16.5	>25	0.66
	$T_{1/2}$				
	3.8	73	72.9	73	15

[a] Microdilution method using Mueller-Hinton broth. Streptococci were tested using Todd-Hewitt broth.
[b] In mg/kg per dose in mice.
[c] Half-life in a 10^{-4} M pH 7.4 phosphate buffer solution at 37°C in hours.
[d] Methicillin resistant strain.

to the tendency of the longer methylene chain (n=4) to fold in aqueous medium thus making the amino group less frequently exposed for reaction.



4 R=H
18 R=$COCH_3$
19 R=$CO(CH_2)_2NH_2$
20 R=$CO(CH_2)_2OH$
21 R=$COCH_2NH_2$
22 R=$COCH_2N_3$
23 R=$CO(CH_2)_2N_3$

Results and Discussion

As anticipated, the chemical stability of **4** ($T_{1/2}$=3.8 hours) was dramatically improved in the *N*-acetyl derivative **18** ($T_{1/2}$=73 hours, Table 1), althought its *in vitro* and *in vivo* antibacterial activities, especially against *Pseudomonas aeruginosa* were drastically diminished. Similar behavior was exhibited by the *N*-(2-aminoethyl) acyl (**19**) and the *N*-(2-hydroxyethyl) acyl (**20**) derivatives (Table 1). In order to restore the antibacterial activities, particularly *in vivo*, while retaining the highly desirable acylation effect on increased chemical stability, the acyl moiety was chosen as an amino acid or its derivative. In such derivatives the amide bond becomes a peptide bond, which may then be cleaved *in vivo* by non-specific peptidases with release of the parent amine, the β-lactam (**4**). The mono- and dipeptide derivatives of the natural amino acids L-glycine (**21**), L-alanine (**11**, **12**) and a derivative of the latter, the β-chloro-L-alanine (**13**, **14**) showed greater chemical stability and similar or even better *in vivo* (PD_{50}) antibacterial activities than the parent amine (**4**) (Table 2). The much higher PD_{50} values (Table 2) of the unnatural D-alanine derivatives (**9**, **10**) suggest that the peptide bond was not recognized, and hence not cleaved by peptidases, thus making them behave more like simple *N*-acyl

Table 2. *In vitro* (minimum inhibitory concentration, μg/ml)[a] and *in vivo* (PD_{50})[b] data for peptide derivatives, and their chemical stability ($T_{1/2}$)[c].

Bacterial species[d]	MIC								
	4	**9**	**10**	**11**	**12**	**13**	**14**	**21**	Imipenem
S. pn A9585	0.13	0.25	0.25	0.125	0.25	0.03	0.5	0.25	0.001
S. py A9604	0.13	0.25	0.25	0.125	0.125	0.03	0.5	0.25	0.001
E. fae A20688	16	32	32	16	4	16	64	32	0.5
S. au A9537	1	2	4	2	2	0.5	2	2	0.007
S. au A15090	2	2	8	2	8	1	8	2	0.015
S. au MR A20699	32	128	128	128	128	1	128	4	32
E. coli A15119	0.008	0.015	0.06	0.125	0.125	0.5	1	0.016	0.125
K. pn A9664	0.03	0.015	0.06	0.03	0.125	0.5	2	0.03	0.125
E. clo A9659	0.25	0.03	0.5	0.5	2	4	128	0.25	0.5
P. mir A9900	1	0.5	2	0.25	2	8	16	0.25	1
P. vul A21559	0.25	0.5	0.5	4	8	0.5	2	0.25	1
P. rett A22424	2	2	8	4	32	2	16	2	1
Ps. ae A9843	0.25	4	16	8	128	32	128	4	1
	PD_{50}								
S. au A15090	15.6	12.5	>25	10.1	12.25	19.2	5.4	16.5	0.48
Ps. ae A9843	0.2	12.5	4.7	0.1	0.15	1.4	0.6	1.1	0.66
	$T_{1/2}$								
	3.8	72.5	73	73	73	73	73	72.5	15

[a] Microdilution method using Mueller-Hinton broth. Streptococci were tested using Todd-Hewitt broth.
[b] In mg/kg per dose in mice.
[c] Half-life in a 10^{-4} M pH 7.4 phosphate buffer solution at 37°C in hours.
[d] For full names, see Table 1.

derivatives (**18**, **19**, **20**). From the excellent PD_{50} values of the natural L-peptide conjugates of the β-lactam **4**, it is apparent that they act as prodrugs by the cleavage of the peptide bonds by some internal hydrolytic mechanism either before or within the periplasmic space liberating the β-lactam and the peptide moieties, the former expressing its antibacterial action by binding to penicillin binding proteins on the surface of the cytoplasmic membrane. Based on this, it was reasoned that if the liberated peptide moiety, instead of being wasted, might be utilized to augment the antibacterial action of the β-lactam, in which case the peptide conjugate might be viewed as a "dual-action" antibiotic with possible synergistic effect. In this context it may be mentioned that β-chloro-L-alanine (BCLA) inhibits alanine racemase,[14,15] a cytoplasmic enzyme involved in peptidoglycan synthesis, which is also the ultimate target for β-lactam antibiotic action. Hence, the BCLA dipeptide derivative (**14**) was synthesized with the anticipation that the liberated dipeptide might actively be transported by peptide permeases into the bacterial cell where it would be cleaved by peptidases to BCLA. It may be emphasized that the antibacterial action of BCLA is restricted to Gram-positive strains.[14,15] Hence, it is pertinent to compare PD_{50} values of the amine **4** and the BCLA dipeptide conjugate **14** only against *Staphylococcus aureus* in order to assess the synergism. It was gratifying to note that the BCLA dipeptide **14** *in vivo* was *ca.* 3 times more active than the parent amine **4** against this organism (Table 2). The low level of synergism was attributed to a very low intracellular concentration of the inhibitor (BCLA) as a consequence of indiscriminate extracellular cleavage of both the peptide bonds linking the β-lactam moiety (**4**), thus prematurely liberating the inhibitor (BCLA) as a monomer which could not be transported into the cell. The PD_{50} value of the BCLA monopeptide **13** against

S. aureus was similar to that of the parent **4**, in accordance with this contention.

It may be commented that a change in charge characteristics from one positive and one negative (zwitterionic) in **4** to a single negative charge in some of the *N*-acyl derivatives may be responsible for the diminished *in vitro* activities for the latter against *Pseudomonas aeruginosa*, as zwitterionic compounds are known[16] to penetrate the outer membrane F porin (OmpF) channels of these species faster than those with one or two negative charges. However, in the peptide derivatives the size, shape and the associated increase in hydrophobicity may contribute to their decreased *in vitro* activities against *Pseudomonas aeruginosa*.

In conclusion, it may be suggested that while constructing a prodrug, it may be possible to utilize the auxiliary part in augmenting the action of the principal-drug-part, thus rendering the prodrug a "dual-action" drug.

Experimental

Solutions were evaporated below 30°C under diminished pressure. ^{1}H NMR spectra were recorded with a Bruker-AC200SY instrument operating at 200 MHz or AMX 400 operating at 400 MHz for protons. Chemical shifts are expressed in ppm relative to TMS, sodium 3-trimethylsilylpropionate-2,2,3,3-d_4 (TSP, for D_2O samples) or to internal $CDCl_3$ (7.24), acetone-d_6 (2.04), HOD (4.81). IR spectra were recorded on a Perkin-Elmer 781 instrument. UV spectra were recorded on a Hewlett Packard 8451A diode array spectrometer. Solvents were reagent or spectrophotometric/chromatographic grade and used directly or dried over 4 Å molecular sieves. THF was dried over sodium/benzophenone and then distilled. Preparative μBondapak C_{18} reverse-phase silica, 125 Å, 55~105 μ was obtained from Waters, Chromatography Division/Millipore Corporation.

General Procedure for the Preparation of *N*-Carboxy Anhydride[17]

In a representative example, to a suspension of β-chloro-L-alanine (1 g, 8.1 mmol) in dry THF (20 ml) at 50°C, under an inert atmosphere, was added trichloromethylchloroformate. The clear solution was stirred for one hour at 50°C, solvent distilled off with water-aspirator at 30°C, ethyl acetate (20 ml) was added to the residue and filtered. Hexane (80 ml) was added to the filtrate with stirring and the white solid was separated, washed with hexane (100 ml) and dried in vacuum over P_2O_5 (0.88 g, 73%). ^{1}H NMR (200 MHz, acetone-d_6) δ: 4.00, 4.09 (AB of ABX, 2H, $C_\beta H_2$), 5.06 (t, 1H, $C_\alpha H$, $J_{\alpha,\beta}$=3.21 Hz). In $CDCl_3$ δ: 3.86 (d, 2H, $C_\beta H_2$, J=4.5 Hz), 4.65 (dt, 1H, $C_\alpha H$, $J_{\alpha,NH}$=0.79 Hz, decomposes on D_2O exchange).

^{1}H NMR of **7** in acetone-d_6, δ: 1.48 (d, 3H, CH_3, J=6.97 Hz), 4.58 (dq, 1H, $C_\alpha H$, $J_{\alpha,NH}$=0.85 Hz).

General Procedure for the Preparation of Mono- and Dipeptide Derivatives of **4** and **5**

In a typical example, **4** (0.44 g, 1.35 mmol) in 0.2 M pH 7.0 buffer (NaH_2PO_4-Na_2HPO_4, 15 ml) at 0°C was adjusted to pH 10.2 with 1 N NaOH and treated with **6** (0.16 g, 1.35 mmol) in dioxane (1.5 ml) while bubbling argon through the solution. When the pH dropped to 7.2, it was readjusted to 10.2 and left there for one hour (Note: in most cases the reaction was finished in 30 minutes or less) to ensure complete reaction. Finally, the pH was adjusted to 6.0 and argon bubbling was continued for 30 minutes to ensure complete flushing of carbon dioxide. The reaction mixture was passed through a column (4.5 × 12.5 cm) of reverse-phase silica (μBondapak C_{18}) packed in CH_3CN and washed thoroughly with cold water. The crude product was eluted with water, lyophilized to a solid and then carefully rechromatographed on reverse-phase silica as above. Compounds **9** and **10** were eluted one after the other, in that order in all cases, with a mixture of water and CH_3CN (93:7, gradient elution) and were obtained as white fluffy solids after lyophilization (**9**: 0.12 g, 22.4%; **10**: 0.065 g, 10.3%).

General Procedure for the Preparation of Mono- and Dipeptide Derivatives Using the Acylating Species **17**

In a representative example, **4** (0.174 g, 0.54 mmol) in 0.2 M pH 7.0 buffer (NaH_2PO_4-Na_2HPO_4,

10 ml) at 0°C was adjusted to pH 10.2 with 1 N NaOH, under argon. This solution was added to a well stirred mixture containing equimolar quantities of **7** (0.062 g, 0.54 mmol) and DMAP (0.065 g, 0.54 mmol) in dioxane (3 ml) in a separate flask at 0°C while bubbling argon through the solution. Whenever the pH dropped, it was readjusted to 10.2 and left at 0°C for one hour to ensure complete reaction (Note: in some instances it may be necessary to leave the reaction at room temperature for an appropriate time). Finally, the pH was adjusted to 6.0 and argon bubbling was continued for 30 minutes to ensure complete flushing of carbon dioxide. The reaction mixture was passed through a column (4.5 × 10 cm) of reverse-phase silica (μBondapak C_{18}) packed in CH_3CN and washed thoroughly with cold water. The crude product was eluted with water, lyophilized to a solid and then carefully rechromatographed on reverse-phase silica, as above. Compound **11** was eluted with a mixture of water and CH_3CN (96:4, gradient elution) and was obtained as a white fluffy solid after lyophilization (0.065 g, 30.7%). Following the same procedure, the monopeptide **9** was converted into the dipeptide **10** in a yield of 30%.

(4*R*,5*S*,6*S*)-6-[1′(*R*)-1′-Hydroxyethyl]-3-(2-cyanoethylthio)-4-[2-(D-alanyl)-aminoethyl]-7-oxo-1-azabicyclo(3.2.0)-hept-2-ene-2-carboxylic Acid (**9**)

Purity 92.9% by HPLC (UV detection at 300 nm) on μBondapak C_{18} column (4 mm × 30 cm), eluant: 3% CH_3CN in pH 6.8 phosphate buffer, flow rate: 1 ml/minute, retention time: 5.57 minutes, UV λ_{max} 300 nm. IR (nujol) 1750 cm^{-1} (C=O, β-lactam). ^1H NMR (200 MHz, D_2O) δ: 1.38 (d, 3H, CH_3, J=6.38 Hz), 1.45 (d, 3H, CH_3, J=7.05 Hz), 1.65~1.84; 1.99~2.13 (m, 2H, CH_2), 2.83~2.92 (overlap, 2H, CH_2CN), 2.93~3.2 (overlap, 2H, SCH_2), 3.32~3.5 (overlap, 3H, CH_2N, H-4), 3.61 (dd, 1H, H-6, $J_{5,6}$=2.65 Hz, $J_{6,1'}$=6.02 Hz), 3.84 (q, 1H, $C_\alpha H$, J_{α,CH_3}=7.05 Hz), 4.31 (m, 1H, H-5), 4.33 (m, 1H, H-1′).

(4*R*,5*S*,6*S*)-6-[1′(*R*)-1′-Hydroxyethyl]-3-(2-cyanoethylthio)-4-[2-(D-alanyl-D-alanyl)-aminoethyl]-7-oxo-1-azabicyclo(3.2.0)-hept-2-ene-2-carboxylic Acid (**10**)

Purity 88.1% by HPLC (UV detection at 300 nm) on μBondapak C_{18} column (4 mm × 30 cm), eluant: 3% CH_3CN in pH 6.8 phosphate buffer, flow rate: 1 ml/minute, retention time: 7.04 minutes, UV λ_{max} 300 nm. IR (nujol) 1750 cm^{-1} (C=O, β-lactam), ^1H NMR (200 MHz, D_2O) δ: 1.37 (d, 3H, CH_3, J=6.39 Hz), 1.43 (d, 3H, CH_3, J=7.21 Hz), 1.57 (d, 3H, CH_3, J=7.09 Hz), 1.64~1.77; 1.98~2.12 (m, 2H, CH_2), 2.83~2.9 (overlap, 2H, CH_2CN), 2.93~3.2 (overlap, 2H, SCH_2), 3.3~3.4 (overlap, 3H, CH_2N, H-4), 3.61 (dd, 1H, H-6, $J_{5,6}$=2.63 Hz, $J_{6,1'}$=5.83 Hz), 4.11 (q, 1H, $C_\alpha H$, J_{α,CH_3}=7.35 Hz), 4.27~4.38 (overlap, 3H, H-1′, H-5, $C_\alpha H$).

(4*R*,5*S*,6*S*)-6-[1′(*R*)-1′-Hydroxyethyl-3-(2-cyanoethylthio)-4-[2-(L-alanyl)-aminoethyl]-7-oxo-1-azabicyclo(3.2.0)-hept-2-ene-2-carboxylic Acid (**11**)

The title compound was eluted with a mixture of water and CH_3CN (97:3, gradient elution) and was obtained as a yellowish fluffy solid after lyophilization in 15.9% yield. Purity 96.5% by HPLC (UV detection at 300 nm) on μBondapak C_{18} column (4 mm × 30 cm), eluant: 5% CH_3CN in pH 6.8 phosphate buffer, flow rate: 0.6 ml/minute, retention time: 7.25 minutes, UV λ_{max} 300 nm. IR (nujol) 1750 cm^{-1} (C=O, β-lactam). ^1H NMR (200 MHz, D_2O) δ: 1.38 (d, 3H, CH_3, J=6.4 Hz), 1.47 (d, 3H, CH_3, J=7.0 Hz), 1.52~1.77; 1.97~2.11 (m, 2H, CH_2), 2.82~2.89 (overlap, 2H, CH_2CN), 2.91~3.17 (overlap, 2H, SCH_2), 3.27~3.43 (overlap, 3H, CH_2N, H-4), 3.59 (dd, 1H, H-6, $J_{5,6}$=2.66 Hz, $J_{6,1'}$=6.06 Hz), 3.91 (q, 1H, $C_\alpha H$, J_{α,CH_3}=7.02 Hz), 4.74~4.96 (overlap, 2H, H-5, H-1′).

(4*R*,5*S*,6*S*)-6-[1′(*R*)-1′-Hydroxyethyl-3-(2-cyanoethylthio)-4-[2-(L-alanyl-L-alanyl)-aminoethyl]-7-oxo-1-azabicyclo(3.2.0)-hept-2-ene-2-carboxylic Acid (**12**)

The title compound was eluted with a mixture of water and CH_3CN (96:4, gradient elution) and was obtained as a yellowish fluffy solid after lyophilization in 9.4% yield. Purity 96.9% by HPLC (UV detection at 300 nm) on μBondapak C_{18} column (4 mm × 30 cm), eluant: 5% CH_3CN in pH 6.8 phosphate buffer, flow rate: 0.6 ml/minute, retention time: 9.1 minutes, UV λ_{max} 300 nm. IR (nujol) 1750 cm^{-1} (C=O, β-lactam). ^1H NMR (200 MHz, D_2O) δ: 1.37 (d, 3H, CH_3, J=6.45 Hz), 1.42 (d, 3H, CH_3, J=7.39 Hz), 1.46 (d, 3H, CH_3, J=8.85 Hz), 1.59~1.75; 1.99~2.14 (m, 2H, CH_2), 2.84~2.89 (overlap, 2H, CH_2CN), 2.92~3.22 (overlap, 2H, SCH_2), 3.3~3.42 (overlap, 3H, CH_2N, H-4), 3.59 (dd, 1H, H-6, $J_{5,6}$=2.56 Hz, $J_{6,1'}$=5.88 Hz), 3.9 (br, 1H, $C_\alpha H$), 4.25~4.34 (overlap, 3H, H-5, H-1′, $C_\alpha H$).

(4*R*,5*S*,6*S*)-6-[1′(*R*)-1′-Hydroxyethyl-3-(2-cyanoethylthio)-4-[2-(β-chloro-L-alanyl)-aminoethyl]-7-oxo-1-azabicyclo(3.2.0)-hept-2-ene-2-carboxylic Acid (**13**)

The title compound was eluted with a mixture of water and CH_3CN (97:3, gradient elution) and was obtained as a yellowish fluffy solid after lyophilization in 37.5% yield. Purity 88.1% by HPLC (UV detection at 300 nm) on μBondapak C_{18} column (4 mm × 30 cm), eluant: 5% CH_3CN in pH 6.8 phosphate buffer, flow rate: 1 ml/minute, retention time: 8.46 minutes, UV λ_{max} 300 nm. IR (nujol) 1750 cm^{-1} (C=O, β-lactam). ^1H NMR (200 MHz, D_2O) δ: 1.37 (d, 3H, CH_3, J=6.36 Hz), 1.65~1.82; 2.02~2.16 (m, 2H, CH_2), 2.81~2.92 (m, 2H, CH_2CN), 2.94~3.02; 3.03~3.21 (m, 2H, SCH_2), 3.26~3.51 (overlap, 3H, CH_2NH, H-4), 3.59 (dd, 1H, H-6, $J_{5,6}$=2.68 Hz, $J_{6,1'}$=6.02 Hz), 3.87~4.04 (m, 2H, $C_\beta H_2$), 4.11 (t, 1H, $C_\alpha H$, $J_{\alpha,\beta}$=4.57 Hz), 4.27~4.38 (overlap, 2H, H-5, H-1′).

(4*R*,5*S*,6*S*)-6-[1′(*R*)-1′-Hydroxyethyl-3-(2-cyanoethylthio)-4-[2-(β-chloro-L-alanyl-β-chloro-L-alanyl)-aminoethyl]-7-oxo-1-azabicyclo(3.2.0)-hept-2-ene-2-carboxylic Acid (**14**)

The title compound was eluted with a mixture of water and CH_3CN (94:6, gradient elution) and was obtained as a yellowish fluffy solid after lyophilization in 6.8% yield. Purity 92.2% by HPLC (UV detection at 300 nm) on μBondapak C_{18} column (4 mm × 30 cm), eluant: 10% CH_3CN in pH 6.8 phosphate buffer, flow rate: 1 ml/minute, retention time: 8.31 minutes, UV λ_{max} 300 nm. IR (nujol) 1750 cm^{-1} (C=O, β-lactam). ^1H NMR (200 MHz, D_2O) δ: 1.37 (d, 3H, CH_3, J=6.4 Hz), 1.66~1.77; 2.06~2.16 (m, 2H, CH_2), 2.83~2.89 (m, 2H, CH_2CN), 2.91~3.04; 3.08~3.21 (m, 2H, SCH_2), 3.32~3.41 (m, 3H, CH_2NH, H-4), 3.58 (dd, 1H, H-6, $J_{6,1'}$=5.88 Hz), 3.78~4.08 (m, 4H, $C_\beta H_2$), 4.17 (t, 1H, $C_\alpha H$, $J_{\alpha,\beta}$=4.61 Hz), 4.27 (dd, 1H, H-5, $J_{4,5}$=7.92 Hz, $J_{5,6}$=2.48 Hz), 4.31 (m, 1H, H-1′), 4.76 (t, 1H, $C_\alpha H$, $J_{\alpha,\beta}$=5.37 Hz).

(4*R*,5*S*,6*S*)-6-[1′(*R*)-1′-Hydroxyethyl-3-(2-cyanoethylthio)-4-[2-(β-chloro-L-alanyl)-aminobutyl]-7-oxo-1-azabicyclo(3.2.0)-hept-2-ene-2-carboxylic Acid (**15**)

The crude reaction mixture obtained from 0.15 g, 0.42 mmol of **5**, was passed through a column (3.5 × 6 cm) of reverse-phase silica (μBondapak C_{18}). The crude title compound was eluted with a mixture of water and CH_3CN (97:3, gradient elution) and was further purified by preparative HPLC using phosphate buffer as eluant. The desalting was effected by passing through reverse-phase silica (μBondapak C_{18}). The title compound was obtained as a white fluffy solid after lyophilization (0.02 g, 10.3%). Purity 99.7% by HPLC (UV detection at 300 nm) on μBondapak C_{18} column (4 mm × 30 cm), eluant: 10% CH_3CN in pH 6.8 phosphate buffer, flow rate: 0.7 ml/minute, retention time: 7.47 minutes, UV λ_{max} 300 nm. IR (nujol) 1750 cm^{-1} (C=O, β-lactam). ^1H NMR (200 MHz, D_2O) δ: 1.35 (d, 3H, CH_3, J=6.39 Hz), 1.45~1.9 (overlap, 6H, $(CH_2)_3$), 2.83~3.37 (overlap, 7H, H-4, CH_2CN, SCH_2, CH_2NH), 3.42 (dd, 1H, H-6, $J_{5,6}$=2.49 Hz, $J_{6,1'}$=5.93 Hz), 3.85 (centre of m, 3H, $C_\alpha H$, $C_\beta H_2$), 4.25 (dd, overlap, 1H, H-5), 4.29 (m, 1H, H-1′).

Sodium (4*R*,5*S*,6*S*)-6-[1′(*R*)-1′-Hydroxyethyl]-3-(2-cyanoethylthio)-4-[2-(acetyl)-aminoethyl]-7-oxo-1-azabicyclo(3.2.0)-hept-2-ene-2-carboxylate (**18**)

A solution of (4*R*,5*S*,6*S*)-6-[1′(*R*)-1′-hydroxyethyl]-3-(2-cyanoethylthio)-4-[2-(amino)ethyl]-7-oxo-1-azabicyclo(3.2.0)-hept-2-ene-2-carboxylic acid **4** (0.26 g, 0.8 mmol) in 0.1 M pH 7.0 sodium-phosphate buffer (10 ml) was cooled to 0°C and dioxane (10 ml) was added. Acetyl chloride (0.19 g, 0.17 ml, 2.4 mmol) was added and the pH was adjusted to 9 with 1 N NaOH, when it dropped to 2.3. After an hour acetic anhydride (0.245 g, 0.23 ml, 2.4 mmol) was added to ensure complete *N*-acetylation and the pH was readjusted to 8 and left there for one hour. The reaction mixture was passed through a column of μBondapak C_{18} reverse-phase silica (4.5 × 11 cm) packed in CH_3CN and washed thoroughly with cold water. Appropriate fractions belonging to the product were eluted with water, combined and lyophilized to a solid which was rechromatographed on reverse-phase silica, as above. The title compound was eluted with water and was obtained as a white fluffy solid after lyophilization (0.07 g, 22.6%). Purity 99.5% by HPLC (UV detection at 300 nm) on μBondapak C_{18} column (4 mm × 30 cm), eluant: pH 7.0 phosphate buffer, flow rate: 1.5 ml/minute, retention time: 10.66 minutes, UV λ_{max} 300 nm. IR (nujol) 1750 cm^{-1} (C=O, β-lactam). ^1H NMR (400 MHz, D_2O) δ: 1.35 (d, 3H, CH_3, J=7.05 Hz), 1.64~1.73; 1.98~2.07 (m, 2H, CH_2), 2.02 (s, 3H, $COCH_3$), 2.82~2.86 (m, 2H, CH_2CN), 2.91~3.12 (m, 2H, SCH_2), 3.2~3.37 (overlap, 3H, CH_2NH, H-4), 3.56 (dd, 1H, H-6, $J_{5,6}$=2.75 Hz, $J_{6,1'}$=6.01 Hz), 4.27 (dd, 1H, H-5, $J_{4,5}$=9.38 Hz),

4.29 (q, 1H, H-1').

2-Azidopropionyl Chloride

Chloropropionic acid (10 g, 0.092 mol) in water (10 ml) was stirred until a clear solution was obtained, and then 3.45 ml of a 2 M NaOH solution was added dropwise over a period of 15 minutes. A slurry of sodium azide (15.6 g, 0.24 mol) in water (20 ml) was added portionwise to this mixture and stirred until a clear solution was obtained, adding more water (6 ml) if needed. Ether (50 ml) was added and the mixture was gently refluxed at 65°C for 24 hours and at 75°C for additional 24 hours, with stirring. The reaction mixture was cooled in ice-water bath and conc H_2SO_4 (4.42 ml in 16.5 ml of water) was added dropwise over a period of one hour. The pH of the aqueous phase was adjusted from 6 to 1 by careful addition of conc H_2SO_4. Sodium chloride was added and the ether layer was decanted from the voluminous solid/gel. More ether (50 ml) was added, stirred and decanted. The process was repeated once more. The combined ether solution was dried over anhydrous Na_2SO_4, and the solvent was removed *in vacuo* to give a syrup which was finally rotary evaporated 3 times with 100 ml portions of CH_2Cl_2 to remove residual ether and the hazardous HN_3 to give 2-azidopropionic acid (10.7 g). IR (neat) 2100 cm^{-1} (N_3). ^1H NMR (200 MHz, $CDCl_3$) δ: 2.62 (t, 2H, CH_2CO, J=6.42 Hz), 3.57 (t, 2H, N_3CH_2).

The above acid (10.7 g, 0.093 mol) was treated dropwise, under argon, with thionyl chloride (22.13 g, 13.57 ml, 0.186 mol) over a period of 15 minutes. After gas evolution abated, the mixture was heated at 55°C for 2 hours, and fractionally distilled. The fraction boiling in the range 76~82°C at 28 mmHg was collected. IR (neat) 1790 cm^{-1} (COCl), 2100 cm^{-1} (N_3). ^1H NMR (200 MHz, $CDCl_3$) δ: 3.11 (t, 2H, CH_2CO, J=6.2 Hz), 3.61 (t, 2H, N_3CH_2).

Sodium (4*R*,5*S*,6*S*)-6-[1'(*R*)-1'-Hydroxyethyl]-3-(2-cyanoethylthio)-4-[2-(azidoethyl-carbonyl)-aminoethyl]-7-oxo-1-azabicyclo(3.2.0)-hept-2-ene-2-carboxylate (**23**)

A solution of **4** (0.26 g, 0.8 mmol) in 0.2 M pH 7.0 sodium phosphate buffer (20 ml) at 0°C was diluted with dioxane (20 ml) and the pH was adjusted to 8.5 with 1 N NaOH. 2-Azidopropionylchloride (0.215 g, 1.61 mmol) in dioxane (2 ml) was added, the pH was readjusted to 8.5 and maintained there for one hour after which it was adjusted to 7.0 with 1 N HCl. The crude reaction mixture was passed through a column (4.5 × 11 cm) of reverse-phase silica (μBondapak C_{18}) packed in CH_3CN and washed thoroughly with cold water. Eluted with water and the fractions belonging to the product were combined and lyophilized to a solid which was rechromatographed on reverse-phase silica, as above. The title compound was eluted with a mixture of water and CH_3CN (96:4, gradient elution) and was obtained as a white fluffy solid after lyophilization (0.194 g, 54.3%). ^1H NMR (200 MHz, D_2O) δ: 1.37 (d, 3H, CH_3, J=6.4 Hz), 1.63~1.85; 1.99~2.14 (m, 2H, CH_2), 2.57 (t, 2H, $COCH_2$, J=6.19 Hz), 2.82~2.89 (m, 2H, CH_2CN), 2.93~3.0; 3.03~3.23 (m, 2H, SCH_2), 3.31~3.42 (m, 3H, CH_2NH, H-4), 3.61 (dd, 1H, H-6, $J_{5,6}$=2.72 Hz, $J_{6,1'}$=6.96 Hz), 3.64 (t, 2H, CH_2N_3), 4.3 (dd, 1H, H-5, $J_{4,5}$=10.01 Hz), 4.32 (m, 1H, H-1').

(4*R*,5*S*,6*S*)-6-[1'(*R*)-1'-Hydroxyethyl]-3-(2-cyanoethylthio)-4-[2-(aminoethylcarbonyl)-aminoethyl]-7-oxo-1-azabicyclo(3.2.0)-hept-2-ene-2-carboxylic Acid (**19**)

A solution of **23** (0.165 g, 0.37 mmol) in a 0.1 M pH 7.0 sodium phosphate buffer (20 ml) was hydrogenated at 0°C over 5% palladium on alumina (1 g) at 3.164 kg/cm^2 for one hour. The catalyst was filtered, washed with water, the pH of the filtrate was adjusted to 6.8 with 1 M NaH_2PO_4 and passed through a column (4.5 × 11 cm) of reverse-phase silica (μBondapak C_{18}). Two compounds were eluted from the column of which the less polar title compound was eluted with a mixture of water and CH_3CN (96:4, gradient elution) and was obtained as a white fluffy solid after lyophilization (0.05 g, 34%). Purity 95.3% by HPLC (UV detection at 300 nm) on μBondapak C_{18} column (4 mm × 30 cm), eluant: 8% CH_3CN in pH 5.8 phosphate buffer, flow rate: 0.5 ml/minute, retention time: 8.9 minutes, UV λ_{max} 300 nm. IR (nujol) 1750 cm^{-1} (C=O, β-lactam). ^1H NMR (200 MHz, D_2O) δ: 1.39 (d, 3H, CH_3, J=6.35 Hz), 1.65~1.84; 2.0~2.14 (m, 2H, CH_2), 2.71 (t, 2H, $COCH_2$, J=6.81 Hz), 2.85~2.93 (m, 2H, CH_2CN), 2.96~3.22 (m, 2H, SCH_2), 3.28 (t, 2H, CH_2NH_2), 3.24~3.43 (overlap, 3H, CH_2NH, H-4), 3.61 (dd, 1H, H-6, $J_{5,6}$=2.56 Hz, $J_{6,1'}$=6.02 Hz), 4.32 (dd, 1H, H-5, $J_{4,5}$=9.51 Hz), 4.34 (m, 1H, H-1').

The more polar compound was eluted with water and was obtained as a white fluffy solid after lyophilization (0.03 g, 19.3%). Purity 96.8% by HPLC (UV detection at 300 nm) on μBondapak C_{18}

column (4 mm × 30 cm), eluant: 3% CH_3CN in pH 7.4 phosphate buffer, flow rate: 0.8 ml/minute, retention time: 5.42 minutes, UV λ_{max} 300 nm. IR (nujol) 1750 cm^{-1} (C=O, β-lactam). ^1H NMR of **20** (200 MHz, D_2O) δ: 1.39 (d, 3H, CH_3, J=6.28 Hz), 1.74~1.79; 2.05~2.11 (m, 2H, CH_2), 2.53 (t, 2H, $COCH_2$), 2.84~3.21 (overlap, 4H, SCH_2, CH_2CN), 3.34~3.44 (overlap, 3H, CH_2NH, H-4), 3.61 (m, 1H, H-6), 3.88 (t, 2H, CH_2OH, J=6.0 Hz), 4.32 (m, 1H, H-5), 4.33 (m, 1H, H-1').

Sodium (4*R*,5*S*,6*S*)-6-[1'(*R*)-1'-Hydroxyethyl]-3-(2-cyanoethylthio)-4-[2-(azidoacetyl)-aminoethyl]-7-oxo-1-azabicyclo(3.2.0)-hept-2-ene-2-carboxylate (**22**)

A solution of **4** (0.26 g, 0.8 mmol) in 0.2 M pH 7.0 sodium-phosphate buffer (20 ml) at 0°C was diluted with dioxane (20 ml) and the pH was adjusted to 8.5 with 1 N NaOH. Azidoacetyl chloride[18] (0.3 g, 2.5 mmol) in dioxane (2 ml) was added, the pH was readjusted to 8.0 and maintained there for one hour after which it was adjusted to 7.0 with 1 N HCl. The crude reaction mixture was passed through a column (4.5 × 12 cm) of reverse-phase silica (μBondapak C_{18}) packed in CH_3CN and washed thoroughly with cold water. Eluted with water and the fractions belonging to the product were combined and lyophilized to a solid which was rechromatographed on reverse-phase silica, as above. The title compound was eluted with a mixture of water and CH_3CN (98:2, gradient elution) and was obtained as a white fluffy solid after lyophilization (0.12 g, 35%). ^1H NMR (200 MHz, D_2O) δ: 1.36 (d, 3H, CH_3, J=6.37 Hz), 1.63~1.82; 1.99~2.13 (m, 2H, CH_2), 2.81~2.89 (m, 2H, CH_2CN), 2.91~2.99; 3.02~3.17 (m, 2H, SCH_2), 3.35 (m centre, 3H, CH_2NH, H-4), 3.59 (dd, 1H, H-6, $J_{5,6}$=2.67 Hz, $J_{6,1'}$=5.94 Hz), 4.06 (s, 2H, CH_2N_3), 4.29 (dd, 1H, H-5, $J_{4,5}$=10.04 Hz), 4.31 (m, 1H, H-1').

(4*R*,5*S*,6*S*)-6-[1'(*R*)-1'-Hydroxyethyl]-3-(2-cyanoethylthio)-4-[2-(glycyl)-aminoethyl]-7-oxo-1-azabicyclo(3.2.0)-hept-2-ene-2-carboxylic Acid (**21**)

A solution of **22** (0.1 g, 0.23 mmol) in 0.1 M pH 7.0 sodium-phosphate buffer was hydrogenated at 0°C over 5% palladium on alumina (0.2 g) at 3.164 kg/cm^2 for one hour. The catalyst was filtered, washed with water, the pH of the filtrate was adjusted to 6.8 with 1 M NaH_2PO_4 and passed through a column (4.5 × 10.5 cm) of reverse-phase silica (μBondapak C_{18}). The title compound was eluted with a mixture of water and CH_3CN (98:2, gradient elution) and was obtained as a white fluffy solid after lyophilization (0.087 g, 98%). Purity 93.4% by HPLC (UV detection at 300 nm) on μBondapak C_{18} column (4 mm × 30 cm), eluant: 8% CH_3CN in pH 6.8 phosphate buffer, flow rate: 0.5 ml/minute, retention time: 6.97 minutes, UV λ_{max} 300 nm. IR (nujol) 1750 cm^{-1} (C=O, β-lactam). ^1H NMR (200 MHz, D_2O) δ: 1.36 (d, 3H, CH_3, J=6.39 Hz), 1.73~1.78; 1.98~2.08 (m, 2H, CH_2), 2.8~2.89 (m, 2H, CH_2CN), 2.92~2.99; 3.02~3.19 (m, 2H, SCH_2), 3.5 (m centre, 3H, CH_2NH, H-4), 3.58 (dd, 1H, H-6, $J_{6,1'}$=6.18 Hz), 3.61 (s, 2H, $COCH_2$), 4.29 (dd, 1H, H-5, $J_{5,6}$=2.67 Hz, $J_{4,5}$=9.0 Hz), 4.3 (m, 1H, H-1').

Acknowledgments

The authors wish to thank Prof. A. I. MEYERS for helpful discussions, Dr. R. A. PARTYKA and Dr. M. MÉNARD for their interest.

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