193. Preparation and Conformational Properties of Benzylpenicilloyl-oligo-L-lysine Conjugates

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Summary

Selected oligo-L-lysine carriers and a poly-L-lysine were penicilloylated with benzylpenicillin. The resulting conjugates **2**–**6** were studied by IR. absorption in the solid state and circular dichroism measurements in solution. The IR. data demonstrate the lack of β-structure formation even in medium-sized peptides where such structures might be expected on the basis of previous studies on differently substituted oligo-L-lysines. Considerable proportions of right-handed α-helical conformation are exhibited by the icosa-L-lysine and poly-L-lysine conjugates **5** and **6** in water and 2,2,2-trifluoroethanol. Difficulties in obtaining fully penicilloylated conjugates are not related to the extent of α-helical conformation in aqueous solution.

1. Introduction. – Oligo-L-lysine conjugates carrying haptenic groups in their side chains are of immunochemical interest because they are efficient anaphylactogens in the specifically sensitized organism. On the other hand they seem able to block lymphocytes producing hapten-specific immunoglobulins and thus induce long-lasting immunological tolerance [1] [2]. Detailed evaluation of these biological activities requires *inter alia* information on the conformational properties of the conjugates. Although conformational studies on the ε-unsubstituted and ε-substituted poly-L-lysines are abundant [3], it is not possible to predict the conformational behaviour of oligo-L-lysines carrying bulky substituents such as penicilloyl groups which are of the size of short peptidyl groups representing immunodominant parts of protein antigenic determinants. It was therefore of interest to take advantage of an ongoing project on synthetic oligo-L-lysine carriers and to prepare and study selected ε-(benzylpenicilloyl) (Bpo) conjugates by IR. absorption and CD. measurements.

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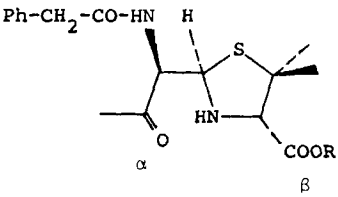
2. Preparation of conjugates. – Penicilloylations of $(Lys)_n$-carriers were performed with sodium benzylpenicillinate at high pH by established procedures (*cf. e.g.* preparation of **1** in [4]) according to *Table 1.* The benzylpenicilloyl substituents I are derivatives of D-benzylpenicilloic acid involving the α-carboxyl group[2]). The α-isomeric form (*cf.* [4]) of the penicilloyl structure is the only one expected to form, but complete stereoisomeric purity with regard to the two chiral C-atoms of the original β-lactam ring does not persist in alkali [5]. Diastereo-



I R = H or any cation

Table 1. *Designation and conditions for the synthesis of Bpo-conjugates*

	Designation[a])	Initial molarity of carrier amino-groups [mol/l]	Benzyl-penicillin added[b]) [mmol/ml]	Reac-tion time [h]	pH	NH_2[c]) [%]
1	6-(benzylpenicilloyl)aminohexanoic acid bis(benzylammonium) salt			*cf.* [4]		
2	Ac-Lys(Bpo)-(Lys(Bpo))$_6$-Lys(Bpo)-O$^-$ · 9 Na$^+$	0.4	0.8 in 3 portions	40	10.5–11	2.5
3	Bpo-Lys(Bpo)-(Lys(Bpo))$_6$-Lys(Bpo)-O$^-$ · 10 Na$^+$	0.8	2.4 in 7 portions	140	10.5–11	6.6
4	CH$_2$(–CO-Lys(Bpo)-(Lys(Bpo))$_3$-Lys(Bpo)-O$^-$)$_2$ · 12 Na$^+$	0.69	1.4 in 2 portions	24	10.5–11	0.0
5	Bpo-Lys(Bpo)-(Lys(Bpo))$_{18}$-Lys(Bpo)-O$^-$ · 22 Na$^+$	0.42	2.7 in 3 portions	96	10.2–10.8	5.0
6	(Lys(Bpo))$_{\overline{40}}$ · $\overline{40}$ Na$^+$ [d])	0.25	0.7 in 3 portions	96	10.5–11	5.0

[a]) In all formulae Bpo represents 1 as its carboxylate anion. [b]) Quantity of benzylpenicillin (in mmol) added per ml of initial oligo-L-lysine solution. [c]) Free amino groups in % of theoretically substitutable amino groups remaining in the isolated final product as determined by the TNBS-method. [d]) This formula depicts an average molecule of a population of polymer homologs.

[2]) According to usual nomenclature the four diastereomers of D-benzylpenicilloic acid are designated α, β, γ and δ. These prefixes are not related to the designation of the α- and β-carboxyl groups in penicilloic acids.

isomerized penicilloyl groups are therefore expected to be present on the conjugates, particularly when long reaction times are involved for their preparation.

Progress of the reaction was controlled by subjecting small aliquots of the reaction solution to ninhydrin or *Fluram* (=4-phenylspiro[furan-2(3*H*),1′-phthalan]-3,3′-dione; *Roche*) color tests and to high voltage paper electrophoresis. A single conjugate, namely **4**, was found to be virtually unstainable by *Fluram* after apparently 'exhaustive penicilloylation'. Its carrier comprises two penta-L-lysine chains linked together *via* their amino termini with a succinic acid bridge *(Table 1)*. Determination of free amino groups in the isolated conjugate by the corresponding to **3** has a reactive N-terminal-α-amino group and seems more difficult to penicilloylate than the octa-L-lysine derivative corresponding to **2** where however, contain several per cent of unsubstituted amino groups. The carrier corresponding to **3** has a reactive N-terminal-α-amino group and seems more difficult to penicilloylate than the octa-L-lysine derivative corresponding to **2** where the chain length is the same but the N-terminus is acetylated. It is in line that the longer conjugates **5** and **6** show an intermediate extent of penicilloylation (95%), whereas the octa-L-lysine derivative **3** is least penicilloylated (93,4%). Indeed, the free α-amino group of the carriers corresponding to **5** and **6** represents a smaller proportion of the total amino groups present. The results of the TNBS-assay also show that the tendency to form α-helical structures in aqueous media as established in this paper for conjugates **5** and **6** (s. below), but not for **2** and **3**, has very little, if any influence on the ease of penicilloylation.

The conjugates appeared homogeneous on thin layer chromatography and high voltage paper electrophoresis. Obviously, these methods do not readily detect small proportions of partially substituted conjugates and products with isomerized penicilloyl groups. This is also true for the data of the elemental analyses and the molar penamaldate values *(Table 2)*. The molar penamaldate value of conjugate **4** (disregarding the monohaptenic compound **1**) is nevertheless among the two highest ones, and it is expected that marked incompleteness of penicilloylation will be

Table 2. *Elemental analysis data*[a]) *and penamaldate assay*[b]) *of Bpo-conjugates*

Conjugate	Molecular formula (molecular weight)	Elemental analysis				Penamaldate assay	
		N		S		PV_m/Bpo[b])	PS_{10} [%]
		Calc.	Found	Calc.	Found		
1	*cf.* [4]					$2.40 \cdot 10^4$	96
2[c])	$C_{178}H_{244}N_{32}O_{42}S_8$ (3761)	11.92	11.36	6.82	7.29	$1.78 \cdot 10^4$	96
3[c])	$C_{192}H_{260}N_{34}O_{45}S_9$ (4053)	11.75	11.81	7.12	7.28	$1.73 \cdot 10^4$	95
4[c])	$C_{224}H_{306}N_{40}O_{54}S_{10}$ (4744)	11.81	11.53	6.76	6.50	$1.78 \cdot 10^4$	98
5	$C_{456}H_{598}N_{82}O_{105}S_{21}Na_{22}$ (10088)	11.39	10.85	6.67	6.29	$1.69 \cdot 10^4$	95
6[c])	$[C_{22}H_{30}N_4O_5S]$ ([462.6])	12.11	12.10	6.93	7.17	$1.55 \cdot 10^4$	98

[a]) Performed by *H. Frohofer*, Institute for Organic Chemistry, University of Zürich. [b]) PV_m/Bpo ($mol^{-1}\,l\,cm^{-1}$) is the molar penamaldate value [7] divided by the theoretical number of Bpo-groups. It is of the order of 16000–18000 for disubstituted and multisubstituted Bpo-derivatives (*cf.* [8]). The value for compound **1** which is a bis(benzylammonium) salt is markedly higher; its Na-salt shows a value around 20000. The penamaldate stability value PS_{10} [8] is $\geq 95\%$ for penicilloic-acid-free Bpo-derivatives. [c]) For the purpose of the elemental analysis, **2**–**4** and **6** were converted to their free acids.

indicated by this assay, particularly by its more precise version presently under study [9].

3. IR. absorption of solid samples. – The positions and relative intensities of the bands of conjugates **2**–**6** *(Table 3)* are very similar and characterized by an amide-I (C=O stretching) vibration at 1649–1654 cm^{-1} [10]. Other common features, although less useful for conformational assignments, are the amide-A (N–H stretching) vibration near 3300 cm^{-1} [11] [12], the vibration near 1595 cm^{-1}, due to the antisymmetrical stretching of the carboxylate group [13] of the Bpo-moieties, and the amide-II vibration at 1530–1540 cm^{-1} [10].

Table 3. *IR. data in the solid state of Bpo-conjugates*

Sample[a])	Region 3500–3200 cm^{-1}[b])	Region 1800–1500 cm^{-1}[b])
1	3400 (*S*), *3293*	*1643*, 1554
2	3400, *3295*	*1649*, 1598, 1532
3	3400, *3295*	*1650*, 1597, 1534
4	3400 (*S*), *3296*	*1649*, 1595, 1535
5	3400 (*S*), *3298*	*1650*, 1594, 1539
6	3400 (*S*), *3305*	*1654*, 1598, 1538

[a]) All samples are carboxylate salts.
[b]) The most intense band of each region is in italics; *S*: shoulder.

It is not possible to discriminate between the α-helical structure, which might be envisaged for the higher homologs **5** and **6**, and an unordered form using the positions of the amide-I band [10]. Of interest, however, is the absence in all conjugates of the 1625–1630 cm^{-1} band, the amide-I vibration of peptide molecules in the β-conformation [11] [12].

4. Solution conformational analysis by CD. – The CD. patterns recorded in H_2O *(Fig.)* and CF_3CH_2OH are similar. The relatively weak positive dichroism between 265–240 nm and the strong positive one below 220–210 nm are interpreted as side-chain contributions since these bands also occur in the model compound **1**. It appears that they represent the dichroic absorption of the thioether chromophore in α-position with respect to a chiral C-atom; this is well-documented for penicillin and other systems [14] [15]. The only peptide band assignable is the negative one at about 220 nm. This band of intermediate intensity is lacking in compound **1**.

The molar ellipticity values per residue at 222 nm ($n \rightarrow \pi^*$ transition of the peptide chromophore) in H_2O are about -3000 for the two octa-L-lysine and the deca-L-lysine conjugates **2**, **3**, and **4**, respectively, approximately -11000 for the icosa-L-lysine conjugate **5**, and about -26000 for the polymeric conjugate **6**. According to the relationship of *Chen & Yang* [16], the last two values imply a right-handed α-helical content of about 30% for the icosapeptide conjugate **5** and about 80% for the polymer **6**. The percentage of α-helix in the shorter conjugates is negligible.

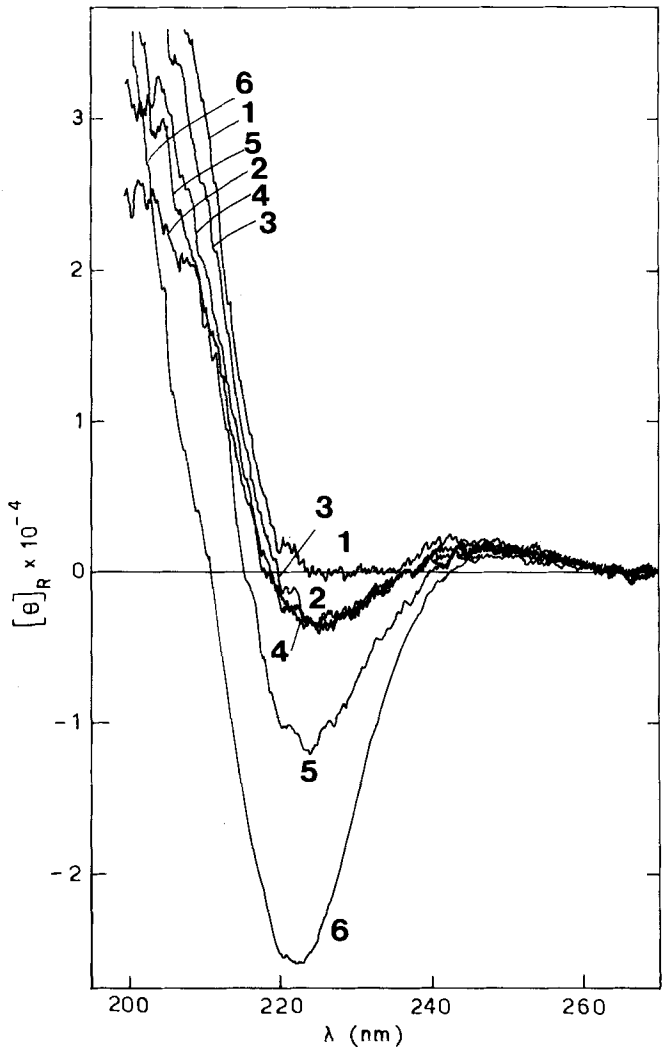


Figure. *Original, computer-drawn CD. spectra of the [Lys(Bpo)]$_n$-peptides in H_2O.* For the structure of **1**–**6**, s. *Table 1.*

In the helix-supporting solvent CF_3CH_2OH, conjugate **4** shows an ellipticity of -3000 and thus does not have any significant α-helical content. In contrast, the ellipticity values in this fluoroalcohol are about -8000 for **2** and **3**, -18000 for **5** and -40000 for **6**. If the latter value is taken as representing a full α-helix (100%), the α-helical contents are 20% for **2** and **3** and 45% for **5**.

5. Discussion. – An interesting result from the solid-state IR. absorption measurements is the lack of evidence for β-structure formation in all [Lys(Bpo)]$_n$-con-

jugates. Since a well-developed β-structure was seen in the $[Lys(Z)]_n$ [11] and $[Lys(Boc)]_n$ [12] homooligopeptide series for n = 5–8, it appears that the Bpo-groups disfavour the regular association of the peptide chains typical of that ordered structure.

With regard to the conformation in aqueous solution of the polymerized $[Lys(Bpo)]_n$, which is an anionic polyelectrolyte, it is well-established that its analog poly-L-glutamic acid is completely non-helical in the 80–100% ionization range while completely helical in the 0–40% ionization range [3]. Charge repulsions are believed to be responsible for the conformational instability. It is also known that increasing helix stabilities of anionic polypeptides are correlated with the increasing number of atoms in their side chains separating the ionizable carboxyl groups from the backbone [17]. This effect can be associated with two factors, namely increased hydrophobic interactions [17] and decreased side-chain charge repulsions [18]. However, in 3-carboxylatopropionyl- or 4-carboxylatobutyryl-substituted poly-L-lysines these factors are not strong enough to bring the substituted polymers into helical conformations in neutral aqueous solution [17]. It is therefore remarkable that 80 and 30% helical content were found for the polymeric conjugate **6** and the icosa-L-lysine conjugate **5**, respectively. Since the pK_a for the β-carboxyl group of benzylpenicilloic acid or of the α-benzylamide of benzylpenicilloic acid is <2 [19], $[Lys(Bpo)]_n$-conjugates are fully ionized polycarboxylate salts at neutral pH.

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Experimental Part

Methods. IR. spectra in the solid state were recorded using a *Perkin-Elmer* model *580* spectrophotometer and the KBr disk technique. The band positions are accurate to ± 1 cm^{-1}. CD. spectra were recorded using a *Cary* model *61* dichrograph equipped with a *Jasco* model *DP-501 N* data processor and cylindrical fused quartz cells of 0.5, 1.0 and 10 mm pathlengths. Doubly distilled water and 2,2,2-trifluoroethanol (*Fluka*, Buchs) were employed as solvents. The values are expressed in terms of $[\theta]_R$ (= residue molar ellipticity).

Materials. Protected oligo-L-lysines up to the decapeptide were prepared stepwise in solution using the two-phase-purification method [20] [21]. The carrier of conjugate **4** was obtained by condensing an N-terminally deblocked pentapeptide with an N-terminally 3-carboxylatopropionyl-substituted pentapeptide using *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride. The icosalysine derivative **5** was obtained by segment condensation from two decapeptide derivatives. Final deprotection depended on the blocking groups involved (mainly Boc, Z and OBzl) and was made with trifluoroacetic acid or liquid HF. Poly-L-lysine · HBr (5000–10000 daltons) was obtained from *Bachem*, Bubendorf. The synthesis and characterization of 6-(benzylpenicilloyl)aminohexanoic acid bis(benzylammonium) salt (**1**) were described previously [4].

Preparation of conjugates. The initial solutions in H_2O of the carrier Na^+-salts were brought to pH > 10 with 2M K_2CO_3, and sodium benzylpenicillinate was then added at RT. *(Table 1).* The pH was kept roughly constant by periodic adjustments with 5N NaOH. After the reaction, the solutions were acidified to pH 8 with HCl-solution, diluted with H_2O to about twice the volume and passed through a *Sephadex-G-25* column with 0.01M phosphate buffer (pH 7.4) containing 0.9% of NaCl. The fractions containing the conjugate, characterized by stable penamaldate values [7], were combined and evaporated *in vacuo* at 30° to colorless residues. They were dissolved in minimal volumes of water and passed through a *Sephadex-G-10* column with water. This last step brought a virtually complete desalting. The fractions with the conjugates were combined and lyophilized.

For the elemental analyses *(Table 2)* most compounds were converted to the free acid by precipitation of an aqueous solution with $KHSO_4$. The precipitates were centrifuged off, washed with water and dried at 25°/0.01 Torr for 1–2 days over P_2O_5. The unconverted conjugate **5** was similarly dried.

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