# Notizen / Notes

## Metal Ion Binding by Dipeptides: Structure of the Product Generated in "Aspartame" Hydrolysis, Sodium *cyclo*(L-α-Aspartyl-L-phenylalanine) Tetrahydrate, Na[*c*(L-Asp-L-Phe)] · $4H_2O$

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Ester cleavage of aspartame (L-α-aspartyl-L-phenylalanine methyl ester, **1**) by equimolar quantities of NaOH is accompanied by intramolecular cyclisation to sodium 3-benzyl-6-(carboxylatomethyl)-2,5-dioxopiperazine (**2**). The solid-state structure of the crystalline tetrahydrate has been determined by single-crystal X-ray diffraction analysis. The cations are arranged in strings, and each sodium atom is in a distorted octahedral environment of six oxygen atoms, including a β-carboxylato (O1) and an oxo function (O4) of two different dioxopiperazines and four water molecules. No nitrogen coordination is observed. Through the ligand bridging (O1, O4) of the metals and a set of hydrogen bonds involving the amide and carboxylate groups and all water molecules a three-dimensional network is established.

Complexation of alkali metal ions by amino acids and proteins plays an important role in many biological processes[1,2,3]. Evidence suggests that aspartate and glutamate are among the principal mediators and protein binding sites for metal ions. Although stability constants for the alkali aspartates and glutamates are small[4], this weak coordination at several sites of a protein can be highly significant, especially for its tertiary structure.

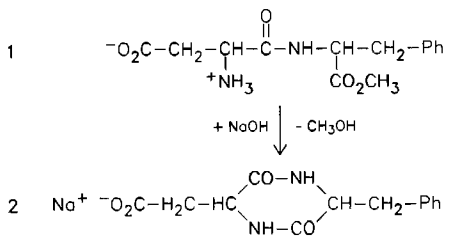
Studies of metal binding to peptides should help to develop a consistent picture of metal binding by proteins and, e.g. in case of cyclic peptides, to understand the activities of cyclic peptide antibiotics[5]. Quite generally metal ion transport in body fluids and through membranes depends strongly on the relative complexing ability of amino acids and their peptides.

As a part of a current programme directed towards investigations of alkali metal binding by amino acids we recently reported (int. al.) on the crystal structure of potassium hydrogen L-glutamate monohydrate[6]. As an extension of this work we studied binding of alkali metal ions to peptides. As a model case we have chosen L-α-aspartyl-L-phenylalanine, a slightly more complicated dipeptide as compared to aspartates or glutamates. Another reason for choosing this peptide was of course its seemingly straightforward synthesis by simple saponification of the corresponding methyl ester, aspartame (**1**). This effective sweetener is widely used as a sugar substitute and a food additive[7].

### Results

For the intended preparation of "sodium aspartame" one equivalent of sodium hydroxide was added to an aqueous solution of L-α-aspartyl-L-phenylalanine methyl ester (**1**) and the mixture stirred for 12 h at ambient temperature. Slow evaporation of the solution over a period of several weeks afforded long colourless needles.

The crystalline product thus obtained was not the expected sodium aspartyl-phenylalaninate but, proven by X-ray diffraction, sodium 3-benzyl-6-(carboxylatomethyl)-2,5-dioxopiperazine (**2**), sodium *cyclo*(L-α-aspartyl-L-phenylalanine), which is not only the product of ester hydrolysis, but also of intramolecular cyclisation. The compound has been characterized by elemental analysis (as the tetrahydrate) and by its NMR spectra.




It has been reported previously that this condensation of the ester hydrolysate is a secondary reaction of the ester cleavage both in neutral and basic medium. The hydrolytic stability of aspartame in aqueous solution is strongly dependent on temperature and on the pH. GLC and HPLC studies have shown that adjusting a 0.05% solution of aspartame with diluted NaOH at room temperature to pH 7 generated two products after several hours: Aspartyl-phenylalanine (AP), due to the base-catalyzed hydrolysis, and the 2,5-dioxopiperazine (DOP) probably also produced by intramolecular aminolysis of the methyl ester linkage[8,9]. While according to these studies AP appears to be the major product, the Na-DOP salt was the only product generated after completion of the crystallisation in the present work. The greater rigidity of the cyclised dipeptide as compared to the more flexible open chain Na-AP, which is less amenable to crystal packing, may be responsible for a complete shift of the equilibrium towards the crystalline Na-DOP · $(H_2O)_4$ precipitate.

### Crystal Structure

The crystal structure of **2** · 4 $H_2O$ has been determined by single-crystal X-ray diffraction analysis at 23°C. The compound forms

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orthorhombic crystals, space group $P2_12_12_1$, with four molecules in the unit cell (Table 1). Figure 1 shows one formula unit and the coordination sphere of the sodium ion which is comprised of four water molecules (O5–O8), a β-carboxylate oxygen atom (O1) and a peptide carbonyl oxygen atom (O4′) of two different neighbouring DOP anions. The coordination geometry corresponds to a distorted octahedron. As indicated by the stoichiometry, all water molecules are thus sodium-coordinated. The remaining oxygen atoms of the DOP (O2, O3) as well as the imino groups (N1, N2) do not participate in metal coordination but are involved in an extensive system of hydrogen bonds. These hydrogen bonds are also cross-linking the individual strings of alternating cations and anions running parallel to the crystallographic *a* axis (Figure 2).

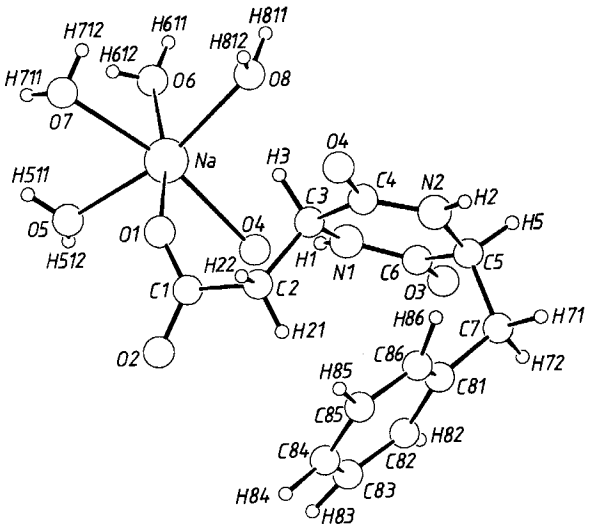



Figure 1. Structure of the *c*(L-Asp-L-Phe)$^-$ anion in **2**·4H$_2$O and the inner coordination sphere of the sodium atom with atomic numbering (arbitrary radii); selected distances [Å] and angles [°]: Na–O1 2.440(2), Na–O4 2.377(2), Na–O8 2.396(2), Na–O5′ 2.471(2), Na–O6′ 2.551(2), Na–O7′ 2.421(2); O1–Na–O4 86.2(1), O1–Na–O8 97.1(1), O4–Na–O8 97.4(1)

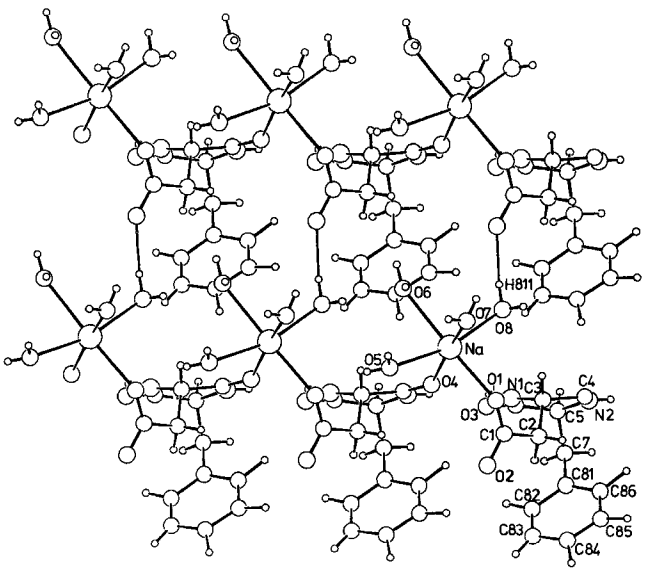



Figure 2. Layer structure of Na[*c*(L-Asp-L-Phe)]·4H$_2$O with atomic numbering (arbitrary radii)

Atomic coordinates are given in Table 1, a list of hydrogen bonds in Table 2. The absolute configuration of the peptide could not be unequivocally confirmed by the attempted refinement with inverse coordinates. It is expected, however, that the configuration of both centers of asymmetry (C3, C5) of the molecule is retained in the dioxopiperazine formation.

Table 1. Fractional atomic coordinates and equivalent isotropic displacement parameters (hydrogen atoms: isotropic displacement parameters) for Na[*c*(L-Asp-L-Phe)]·4H$_2$O (**2**·4H$_2$O); $U_{eq} = \sum_i \sum_j U_{ij} a_i^* a_j^* a_i a_j$

| ATOM | X/A | Y/B | Z/C | U(eq.) |
|---|---|---|---|---|
| NA | 0.0281(2) | 0.7872(1) | 0.1996(0) | 0.313 |
| O1 | 0.2957(3) | 0.5671(2) | 0.2028(0) | 0.315 |
| O2 | 0.2580(3) | 0.3103(2) | 0.1858(0) | 0.516 |
| O3 | 0.2130(3) | 0.6682(2) | 0.0403(0) | 0.305 |
| O4 | 0.9237(2) | 0.6789(2) | 0.1361(0) | 0.277 |
| O5 | 0.2947(4) | 0.1679(2) | 0.2659(1) | 0.374 |
| O6 | 0.2545(4) | 0.4811(2) | 0.7913(1) | 0.600 |
| O7 | 0.3723(3) | 0.1560(3) | 0.7689(1) | 0.444 |
| O8 | 0.3053(5) | 0.9700(3) | 0.1757(1) | 0.672 |
| N1 | 0.3591(3) | 0.6571(3) | 0.1027(1) | 0.269 |
| N2 | 0.7783(3) | 0.6813(2) | 0.0735(1) | 0.250 |
| C1 | 0.3433(4) | 0.4469(3) | 0.1814(1) | 0.220 |
| C2 | 0.5260(4) | 0.4642(3) | 0.1504(1) | 0.243 |
| C3 | 0.5380(4) | 0.6323(3) | 0.1313(1) | 0.212 |
| C4 | 0.7629(4) | 0.6644(3) | 0.1132(1) | 0.265 |
| C5 | 0.6035(4) | 0.6549(3) | 0.0441(1) | 0.204 |
| C6 | 0.3760(4) | 0.6590(3) | 0.0627(1) | 0.252 |
| C7 | 0.6457(5) | 0.5001(3) | 0.0196(1) | 0.271 |
| C81 | 0.6639(4) | 0.3500(3) | 0.0449(1) | 0.325 |
| C82 | 0.4851(5) | 0.2460(3) | 0.0501(1) | 0.479 |
| C83 | 0.4992(7) | 0.1132(3) | 0.0753(1) | 0.675 |
| C84 | 0.6937(8) | 0.0840(3) | 0.0962(1) | 0.770 |
| C85 | 0.8738(6) | 0.1823(4) | 0.0910(1) | 0.738 |
| C86 | 0.8593(5) | 0.3146(3) | 0.0649(1) | 0.575 |

Table 2. Hydrogen bonds [Å] in the crystal structure of Na[*c*(L-Asp-L-Phe)]·4H$_2$O (**2**·4H$_2$O)

| A–B···C | A–B | B···C | A···C | Symmetry operation[a] |
|---|---|---|---|---|
| O5–H511···O2 | 0.946 | 1.996 | 2.917 | a |
| O5–H512···O1 | 0.918 | 1.966 | 2.814 | b |
| O6–H611···O7 | 1.009 | 1.938 | 2.883 | a |
| O6–H612···O1 | 0.989 | 2.016 | 2.978 | a |
| O7–H711···O2 | 0.957 | 1.867 | 2.789 | a |
| O7–H712···O5 | 1.084 | 1.932 | 2.854 | a |
| O8–H811···O2 | 0.954 | 1.977 | 2.849 | c |
| O8–H812···O6 | 1.070 | 1.883 | 2.950 | d |
| N1–H1···O4 | 0.993 | 1.881 | 2.867 | b |
| N2–H2···O3 | 0.932 | 1.935 | 2.861 | d |

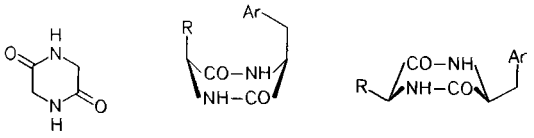
[a] a: $-x, y + 0.5, -z + 0.5$; b: $1 - x, y, z$; c: $x, y + 1, z$; d: $x + 1, y, z$.

## Discussion

2,5-DOP's are present in a number of molecules with important biological activity. Cycloserine, e.g., has been found to be effective against mycobacteriological tuberculosis 30 years ago[10].

Therefore, a large variety of 2,5-DOP's with different *cis* α-carbon substituents have been the subject of extensive studies by NMR spectroscopy, crystal structure determination, and optical rotatory dispersion[11]. The simplest cyclic dipeptide – cyclic diglycine (**3a**) – was found to exist in a planar ring conformation according to an X-ray analysis[12], in agreement with theoretical work focussing on minima of nonbonded interaction[13].

Replacement of one or both glycines by an amino acid with a larger group may change this conformation greatly. Studies on 2,5-DOP's with an aryl substituent (Ar) revealed three possible conformations of the DOP ring: in addition to the planar form, a *flagpole boat* conformation (**3b**) with the *cis* α-carbon substituents in quasi-axial and a *bowsprit boat* conformation with the *cis* α-carbon substituents in quasi-equatorial positions (**3c**).



**3a** **3b** **3c**

The folded conformation with the aromatic ring face-to-face with the DOP ring (**3b**) seems to be most common for cyclic dipeptides containing an aromatic side chain. It was first proposed to be responsible for the ring-shielding effects observed in the NMR spectra of *cyclo*(glycyl-L-phenylalanine)[14], *cyclo*(D-alaninyl-L-phenylalanine), and *cyclo*(glycyl-L-tyrosine)[15]. The form **3b** is found to be less favoured for non-aromatic substituents, e.g. *cyclo*(glycyl-L-valine)[16]. Attractive forces between the two rings may play an important role[17].

The anion of *cyclo*(L-aspartyl-L-phenylalanine) shows the characteristic folding with the two rings facing each other. Surprisingly, the DOP ring is nearly planar, however, with the sums of angles at N1, N2, C4, and C6 of 359.7, 359, 359.9, and 359.9°, respectively, a pattern similar to that of *cyclo*(L-serinyl-L-tyrosine)[15]. In the condensation product of serine, $HO_2C-C(H)NH_2CH_2OH$, and aspartic acid the remaining acidic groups avoid contact with the DOP ring by occupying quasi-equatorial postions, with the amide planarity fully retained. Crystal packing and participation of the acidic groups in hydrogen bonds are certainly also conformation-determining factors.

The structures of only very few metal complexes of cyclic dipeptides are known. In the *cyclo*-disarcosyl complexes with silver – (μ-2-cyclosarcosylsarcosine-*O,O'*)-bis[(μ-3-nitrato-*O,O',O''*)-silver(I)][18] – and lithium – cyclodisarcosyl lithium perchlorate[19] – the metal ions are coordinated to the oxo groups, whereas in bis[cyclodisarcosylhexaaquacopper(II)] perchlorate[20] the cation is surrounded by six water molecules. As expected, in μ-2-*cyclo*(L-methionyl-L-methionyl)-*S,S'*-silver(I) perchlorate dihydrate[21] the silver is attached to the softer sulfur centers.

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## Experimental

All experiments were carried out with bidistilled water. Reagents were of p.a. quality. – pH values: Knick apparatus. – Elemental analyses: Microanalytical laboratory of this Institute. – $^{13}C$- and $^1H$-NMR: Jeol CX 400 spectrometer, dioxane (δ = 67.3) as the internal standard.

*Sodium 3-Benzyl-6-(carboxylatomethyl)-2,5-dioxopiperazine Tetrahydrate* (**2** · 4 $H_2O$): L-α-Aspartyl-L-phenylalanine methyl ester (**1**) (20.67 g, 70.23 mmol), dissolved in 100 ml bidistilled water, was treated with one equivalent of 0.1 M NaOH and stirred overnight. Slow evaporation of the solvent over a period of several weeks afforded colourless needle-shaped crystals (19.6 g, 78% yield). The product is soluble in water to give solutions of pH 7.52. – $^{13}C$ NMR ($D_2O$): δ = 37.46 [d, *J*(CH) = 135.04 Hz, C5], 40.14 [d, *J*(CH) = 136.57 Hz, C3], 51.36 [d, *J*(CH) = 144.97 Hz, C7], 54.68 [d, *J*(CH) = 147.25 Hz, C2], 127.06 [d, *J*(CH) = 161.74 Hz, C84], 128.04 [d, *J*(CH) = 158.69 Hz, C82/86], 129.59 [d, *J*(CH) = 151.82 Hz, C83/85], 133.69 (s, C81), 167.51 (s, C6), 168.46 (s, C4), 176.39 (s, C1). – $^1H$-NMR ($D_2O$): δ = 0.57 (dd, $^2J$ = 16.48, $^3J$ = 10.40 Hz, 1H, H21), 2.07 (dd, $^2J$ = 16.48, $^3J$ = 3.05 Hz, 1H, H22), 2.93 (dd, $^2J$ = 14.04, $^3J$ = 4.27 Hz, 1H, H71), 3.18 (dd, $^2J$ = 14.04, $^3J$ = 4.27 Hz, 1H, H72), 3.99 (dd, $^3J$ = 10.01, $^3J$ = 1.83 Hz, 1H, H3), 4.34 (dd, $^3J$ = 4.27 Hz, 1H, H5), 7.21 (m, H82-H86). The multiplet signal at δ = 0.6 (1H) can be assigned to one of the methylene protons next to the α-carboxylato group. Its upfield shift is a consequence of the ring current in the phenyl group as observed in *cyclo*(glycinyl-L-phenylalanine)[14]. The corresponding signal for "metal free" *cyclo*(L-aspartyl-L-phenylalanine) has a chemical shift of δ = 1.4.

$C_{13}H_{21}N_2NaO_8$ (357.3)
Calcd. C 43.70 H 5.92 N 7.84
Found C 43.71 H 5.91 N 7.85

*Determination of the Crystal Structure:* Suitable crystals of Na[*c*(L-Asp-L-Phe)] · 4 $H_2O$ (**2** · 4 $H_2O$), obtained from an aqueous solution by slow evaporation, were sealed into glass capillaries and mounted on a diffractometer. The crystal data and details of the structure solution procedure are given in Table 3. The data were corrected for Lp effects, but correction for absorption and decay (−0.3%) appeared to be unnecessary. The structure was solved by direct methods (SHELXS-86)[22] and completed by Fourier syntheses. All H atoms with the exception of the phenyl protons could be located in difference syntheses and were included in the final refinement cycles using isotropic displacement parameters (SHELX-76)[23]. The absolute configuration could not be confirmed by refinement of the inverse data set since the *R* values did not give a significant difference, presumably due to the very light alkali ion as well as to a symmetrically balanced electron density[24].

Table 3. Crystal structure data for Na[*c*(L-Asp-L-Phe)] · 4$H_2O$ (**2** · 4$H_2O$)

| |
|---|
| $C_{13}H_{21}N_2NaO_8$; $M_r$ = 357.305; orthorhombic, $P2_12_12_1$ (No. 19); *a* = 6.062(1); *b* = 8.627(1); *c* = 33.175(3) Å; *V* = 1662.55 Å$^3$; *Z* = 4; $d_{calc}$ = 1.42 g/cm$^3$; μ(Mo-$K_\alpha$) = 1.31 cm$^{-1}$; λ = *F*(000) = 624 e; *T* = 23°C; diffractometer: Enraf-Nonius CAD4; radiation: Mo-$K_\alpha$; 0.71069 Å; monochromator: graphite; scan: Θ/2Θ; scan width (in ω): 0.8 + 0.35 tan Θ; $(\sin\Theta/\lambda)_{max}$ 0.59; *hkl* range: ±7, +9, +39; measured reflections: 3256; unique reflections: 2895; $R_{int}$ = 0.0176; refined parameters: 281; observed reflections: 2504 [$F_o > 4.0\sigma(F_o)$], $R^{a)}$ = 0.033, $R_w{}^{b)}$ = 0.027; $\Delta\varrho_{fin}$(max/min) = +0.28/−0.29 e/Å$^3$ |

a) $R = \Sigma(\| F_o \| - \| F_c \|)/\Sigma F_o$. – b) $R_w = [\Sigma(\| F_o \| - \| F_c \|)^2/\Sigma w F_o^2]^{1/2}$, $w = 1/\sigma^2(F_o)$; function minimized: $\Sigma w(\| F_o \| - \| F_c \|)^2$.

CAS Registry Numbers

**1**: 22839-47-0 / **2**: 136983-81-8 / **2** · 4 $H_2O$ (hydrate 1): 136983-82-9 / **2** · 4 $H_2O$ (hydrate 2): 136983-84-1

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[24] Further details of the crystal structure investigations are available on request from the Fachinformationszentrum Karlsruhe, Gesellschaft für wissenschaftlich-technische Information mbH, D-7514 Eggenstein-Leopoldshafen 2, on quoting the depository number CSD-55588, the names of the authors, and the journal citation.

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