4'-(1-Azi-2,2,2-trifluoroethyl)phenylalanine, a Photolabile Carbene-Generating Analogue of Phenylalanine

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Abstract: The synthesis and some of the photochemical properties of 4'-(1-azi-2,2,2-trifluoroethyl)phenylalanine (**7**, ATEPhe), a photolabile analogue of phenylalanine, are described. Upon photolysis with wavelengths >300 nm, **7** rapidly eliminates N_2, yielding a highly reactive carbene capable of OH and CH insertion reactions. The suitability of the title compound and its N- and C-terminal protected derivatives for peptide synthesis is demonstrated by the preparation of analogues of Aspartame (Asp-Phe-OMe) and Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu), in which the original phenylalanine is replaced by its photolabile derivative **7**. Whereas the dipeptide does not exhibit a sweet taste, the pentapeptide shows a biological activity comparable to that of Leu-enkephalin. **7** should be a useful tool for photoaffinity labeling studies on biologically active peptides.

Photoaffinity labeling is a frequently used technique for studying the interactions of biologically active molecules.[1a,b] A photolabile analogue of the compound under investigation, which is stable in the dark, is converted by illumination into a reactive intermediate. This, by forming a covalent bond, stabilizes the interaction based on biological affinity. As precursors aliphatic and especially aromatic azides are used,[2] which on irradation form nitrenes or, most probably, less reactive secondary products.[3] For the more reactive carbenes, diazo compounds have been used as precursors.[4a-c] Unfortunately, their photolysis with wavelengths >300 nm occurs rather slowly with half-life times in the range of hours and often leads to undesired intramolecular rearrangements.

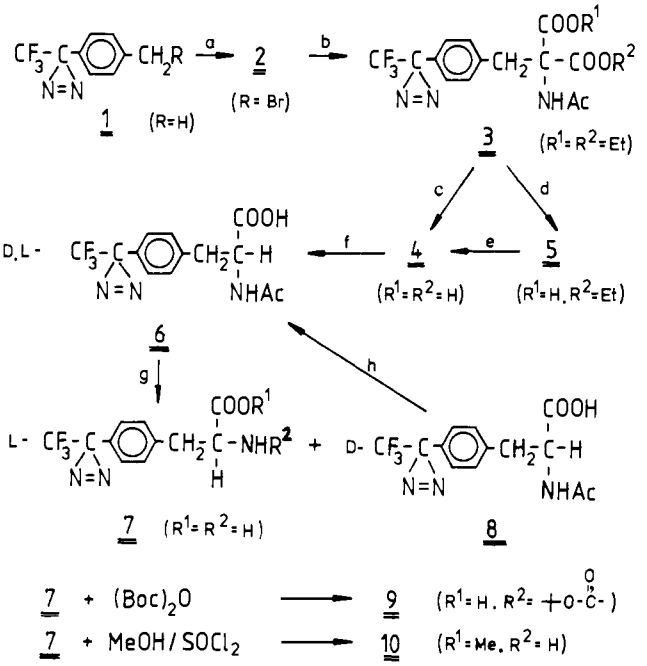
Diazirines[5a,b] share the advantages of both groups: they are photolyzed within minutes at wavelengths around 350 nm and they generate the more reactive carbenes. Analogously to 3-phenyl-3-(trifluoromethyl)-3*H*-diazirine, introduced by Brunner et al.,[6a,b] we recently have synthesized 4-(1-azi-2,2,2-trifluoroethyl)benzoic acid,[7] which via activation of its carboxylic group is easily attached to biomolecules containing amino functions. The compound bound to iodocyanopindolol has been applied successfully to the characterization of β-adrenergic receptors in turkey red cells and guinea pig lung membranes.[8] For investigations dealing with biologically active peptides, the synthesis of the title compound seemed particularly interesting. Its introduction instead of Phe or Tyr should lead only to slight structural changes, and therefore only minor biological alterations would be expected. Additionally, the compound should be valuable as a carbene-generating alternative to *p*-azido-Phe, described some years ago by Wieland[9a] and Schwyzer.[9b]

Results and Discussion

Synthesis. Due to the expected lability of the diazirine moiety its introduction into the amino acid skeleton was originally planned as a late step in the synthesis.

4-Bromotoluene was converted into 2,2,2-trifluoro-1-(4-methylphenyl)-1-ethanone as described.[7] Bromination with NBS gave 1-(4-(bromomethyl)phenyl)-2,2,2-trifluoro-1-ethanone, which via condensation with diethyl acetamidomalonate, saponification to the half ester, and decarboxylation yielded D,L-*N*-acetyl-4'-(trifluoroacetyl)phenylalanine ethyl ester. Prior to conversion of the ketone into the corresponding oxime (precursor of the diazirine analogously to Zeifman et al.[10]) with hydroxylamine hydrochloride, the ethyl ester was saponified to prevent formation of the hydroxamic acid. However, attempts to subsequently tosylate the oxime failed (no reaction at room temperature, at least five new products in refluxing pyridine[11]), probably due to the presence of the carboxylic acid function. An alternative strategy, starting from 3-(4-methylphenyl)-3-(trifluoromethyl)-3*H*-diazirine (**1**), proved successful (see Scheme I).

Scheme I[a]



[a] (a) 1 equiv of NBS, AIBN, reflux; (b) diethyl acetamidomalonate, sodium ethoxide; (c) 4 equiv of aqueous NaOH, dioxane, 16 h, room temperature; (d) 4 equiv of aqueous NaOH, dioxane, 1 h, room temperature; (e) 2.5 equiv of aqueous NaOH, dioxane, 12 h, room temperature; (f) dioxane, 1 h, 80 °C; (g) acylase from *Aspergillus*; (h) excess acetic anhdride in aqueous NaOH.

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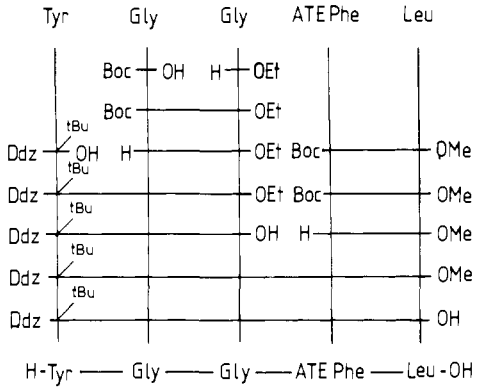
Scheme II



1 was brominated with 1 equiv of NBS in tetrachloromethane at 70–75 °C with AIBN as initiator to give about 70% of the ((bromomethyl)phenyl)diazirine **2** (**2** might be useful for the introduction of the photolabile residue into biomolecules containing amino, hydroxy, or mercapto functions via alkylation). Despite being subjected to column chromatography, **2** was contaminated with small amounts of the dibromobenzyl derivative. This side reaction might be avoided (at the cost of a lower yield) by using less than 1 equiv of NBS. Since the product from the condensation of **2** with the sodium salt of diethyl acetamidomalonate was easily purified by crystallization, complete separation of mono- and dibrominated products by chromatography was not attempted. The diester **3** was saponified with 5 equiv of 2 N aqueous NaOH in dioxane to yield the dicarboxylic acid **4**. This, on heating to 80 °C in dioxane gave nearly pure D,L-*N*-acetyl-4'-(1-azi-2,2,2-trifluoroethyl)phenylalanine (**6**) within 1 h. Prolonged heating or higher temperatures led to several byproducts.

Likewise, when the monoester **5**, obtained by partial saponification of **3**, was heated, side reactions took place before the desired decarboxylation was complete. Stereospecific deacetylation of **6** with acylase I from porcine kidney proceeded very slowly despite the high specific activity (4000 U/mg), probably due to the low affinity of the porcine enzyme toward aromatic amino acids.[12] Instead the acylase from *Aspergillus*[13] (specific activity, 66 U/mg) was used in similar amounts and gave within a few hours the D-*N*-acetyl- (**8**) and the free L-amino acid **7**. Both were separated, after acidification of the aqueous reaction mixture, by extraction of the N-protected D-amino acid into ethyl acetate and chromatography of the concentrated aqueous phase on a Lobar RP-8 reverse phase column using a 0.1% aqueous TFA/methanol gradient as eluent. **8** can be racemized with excess acetic anhydride and again be subjected to the enzymatic digestion. Reaction of **7** with di-*tert*-butyl pyrocarbonate[14] yielded the corresponding Boc-protected derivative **9**. Treatment with thionyl chloride in methanol[15] led to the formation of the methyl ester **10**. Both derivatives should be suitable for peptide synthesis. This was demonstrated by the coupling of **10** to L-Boc-Asp(*t*-Bu) by the MA method.[16] Cleavage of the Boc group and the *tert*-butyl ester by 50% TFA in dichloromethane yielded the free dipeptide methyl ester **12**, a photolabile analogue of the sweetener Aspartame. The same product was obtained by coupling **7** to the

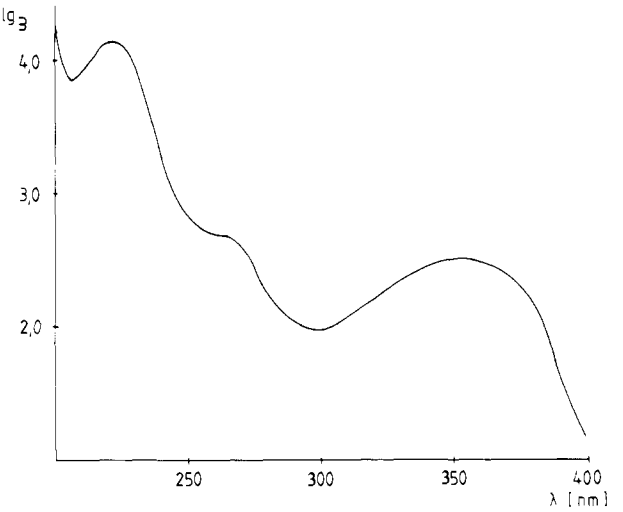


Figure 1. UV spectrum of **16** in H_2O.

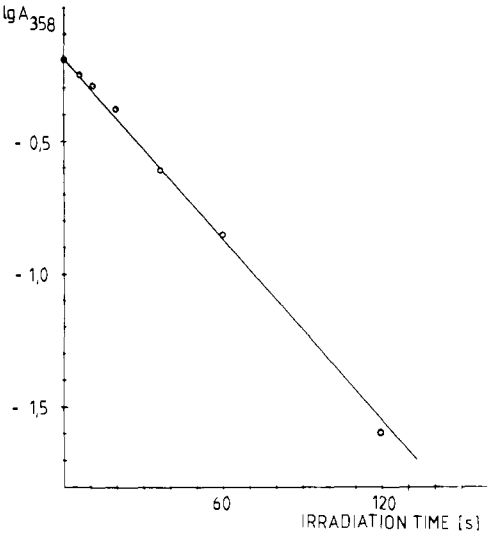


Figure 2. Logarithmic plot of the absorption decrease at 358 nm of **18** upon irradiation (c = 1.924 mM in ethanol) indicating first-order kinetics.

N-hydroxysuccinimide ester of L-Ddz-Asp(*t*-Bu),[17] esterification with diazomethane, and subsequent cleavage of the protective groups as above. Although **12** did not exhibit a sweet taste, its synthesis showed that the diazirine moiety could withstand common peptide chemical reaction conditions. According to results of Goodman et al.[18] substitution of the *p*-hydrogen in the aromatic ring might be crucial for the sweet taste: L-Asp-L-Tyr-OMe is less sweet than Aspartame, methylation of the phenolic OH group leads to even more diminished sweetness; on the other hand, *o*-methoxylation gives a product with the same sweetness as the parent compound. For investigations on sweet-taste receptors, a phenylalanine analogue having the 1-azi-2,2,2-trifluoroethyl residue in the ortho position might be suitable, which could possibly be synthesized by the method described above starting from 2-bromotoluene. **9** was used in the synthesis of a photolabile analogue of Leu-enkephalin[19] (see Scheme II).

By means of the MA method the two fragments Ddz-Tyr(*t*-Bu)-Gly-Gly-OEt and Boc-ATEPhe-Leu-OMe were prepared and coupled by using DCC/HOBt after cleavage of the ethyl ester and the Boc group. Finally, removal of the protective groups yielded the free pentapeptide Tyr-Gly-Gly-ATEPhe-Leu, the

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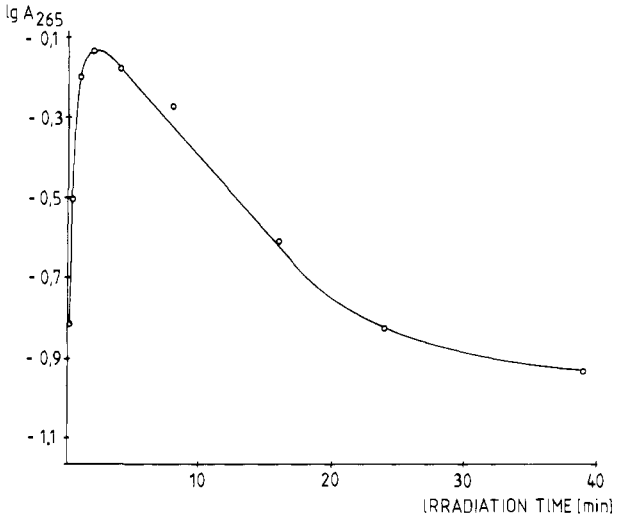


Figure 3. Logarithmic plot of the absorption increase and subsequent decrease at 265 nm during irradiation of **18** (c = 0.0962 mM in ethanol) due to the photochemical rearrangement of the diazirine to the diazo isomer and the photolysis of the latter.

purity of which was checked by HPLC. As determined by the concentrations needed to half maximally inhibit the electrically stimulated contraction of guinea pig ileum and mouse vas deferens, the photolabile analogue is as biologically active as Leu-enkephalin.[20]

Photochemical Properties. All the diazirine-containing compounds described here show the characteristic absorption around 360 nm (see Figure 1).

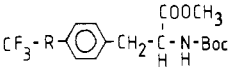
For determination of the time course of the photolysis, an ethanolic solution of **9** (c = 1.924 mM) in a Pyrex screwcap glass tube (filter for wavelengths <300 nm) was irradiated with a Philips HPK 125 W/L high-pressure mercury lamp as described.[7] The diazirine absorption at 358 nm decreases with a half-life period of 27 s following first-order kinetics (see Figure 2).

On the other hand, the absorption at 265 nm strongly increases during the first 2 min and a weak new maximum arises at 460 nm. Both then slowly decrease (half-life period about 10 min; see Figure 3).

As described for other diazirines,[6a,7,21] these changes are based on a photochemical rearrangement of the diazirine into the diazo isomer with subsequent photolysis of the latter. A rearrangement rate of about 30% is obtained by taking the extinction coefficients of 4-(1-diazo-2,2,2-trifluoroethyl)benzoic acid[7] at 284 nm (ϵ 22 000) and 460 nm (ϵ 68) as approximate values for the photoisomerization product of **9**. Following the arguments of Brunner et al.,[6a] the presence of the diazo isomer should lead only to a slight decrease of the cross-linking yield, but not to undesired non-photochemical side reactions giving unspecific labeling (e.g., by an enzymatically acid-catalyzed decomposition of the diazo function; diazo groups with an adjacent trifluoromethyl group are rather stable toward acids[4b]).

To verify the suitability of the new amino acid for photoaffinity labeling, solutions of the *N*-Boc-protected derivative **9** in ethanol, *tert*-butyl alcohol and cyclohexane (c = 2 mM) were irradiated for 30 min under an air atmosphere (Rayonet photoreactor, 16 RPR 3500-Å lamps). TLC showed that in all solvents several products had been formed, the fastest migrating compounds giving a purple color reaction with ninhydrin. Their R_f values were similar to **9** but slightly increasing from ethanol to *tert*-butyl alcohol and cyclohexane (solvent C). Two additional spots with lower R_f values were visible: the upper one showing a brownish, the lower one again a purple color with ninhydrin (identical in the case of all three solvents, but most intense in cyclohexane). Subsequent to treatment with diazomethane, the structures of the products were elucidated by GC/MS and, in order to detect the molecular ions, by field desorption MS (FDMS).

Chart I. Products Formed by Irradiation of 9 in EtOH, *t*-BuOH, and Cyclohexane



14, R = -CH(OC_2H_5)-

15, R = -CH(OC_4H_9)-

16, R = -(C=O)-

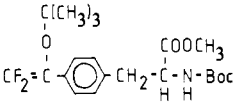
17, R = -CH(OH)-

18, R = -CH(H)-

19, R = -CH(C_6H_{11})-

The main product in ethanol is the ethyl ether **14**, in *tert*-butyl alcohol the *tert*-butyl ether **15** (m/z 405 and 433 in the FD mass spectra), corresponding to the largest peaks (74% and 59%) in the gas chromatograms. In the electron impact mass spectra both molecules give rise to a similar fragmentation pattern, differing by a mass of 28 (corresponding to the difference between C_2H_5 and C_4H_9). Since no loss of H_2O was observed, and on TLC the products showed a lipophilicity similar to or higher than the parent diazirine, they are most likely formed by insertion of the photogenerated carbene into the OH bond of the alcoholic solvents.

The minor byproducts formed in ethanol and *tert*-butyl alcohol showed the same retention times on GC and identical fragments (GC/MS) as well as molecular ions (FDMS; m/z 375 and 377) in their mass spectra. They correspond to the ketone **16** and the alcohol **17** (see Chart I). In ethanol, a small amount (<5%) of the reduced compound **18** (m/z 361) could be detected. The mixture formed in *tert*-butyl alcohol contained an additional major component (22%; longest retention time of the peaks resolved by GC; m/z 413), resulting from insertion into *tert*-butyl alcohol accompanied by elimination of one HF molecule, which is remarkable in view of the high stability of the CF bond:



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Since the molecular ion m/z 413 is present in the FD mass spectrum of the mixture, it is not an artefact formed on the GC column. A similar observation has been reported by Gupta et al.[22] for the photolysis of multilamellar dispersions of phospholipids carrying the 3,3,3-trifluoro-2-diazopropionyl residue;[4b] in contrast, this type of reaction did not occur in unilamellar vesicles. A plausible explanation for the generation of **20** needs further investigation.

Irradiation of **9** in cyclohexane led to about 47% of the CH insertion product **19** (FDMS, m/z 443; GC/MS, fragment m/z 83, corresponding to $C_6H_{11}^+$, with a relative intensity of 62%), together with **16** (34%) and **17** (18%). Small amounts of **18** as well as cyclohexanone (m/z 98) and, tentatively, dicyclohexyl ether (m/z 182) could also be detected. Purging the solution of **9** in cyclohexane with argon prior to photolysis strongly increased the percentage of the CH insertion product **19**, at the cost of both **16** and **17**. Table I summarizes the above results.

Insertion into OH bonds of alcohols,[23] in this case yielding **14** and **15**, is supposed to be characteristic of singlet carbenes,[24]

(20) The pharmacological tests were performed in the laboratory of Prof. A. Herz, Max-Planck-Institut für Psychiatrie, 8000 Munich, FRG. Concentrations necessary for half-maximal inhibition (IC_{50}), [nM] (guinea pig ileum/mouse vas deferens): (a) normorphine (230/710); (b) [D-Ala^2,D-Leu^5]enkephalin (35/0.7); (c) Leu-enkephalin (240/15); (d) **13** (370/16).

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(23) See, for example: Kirmse, W.; Loosen, K.; Sluma, H.-D. *J. Am. Chem. Soc.* **1981**, *103*, 5935–5937 and references therein.

Table I. Relative Abundancies of Photolysis Products of **9** in EtOH, *t*-BuOH, and Cyclohexane[a]

		products,[b] %			
compd	m/z[c]	EtOH	*t*-BuOH	cyclohexane	cyclohexane[d]
14	405	73			
15	433		59		
16	375	17	9	34	18
17	377	6	10	18	9
18	361	5	trace	trace	n.d.[e]
19	443			47	73
20	413		22		

[a] 2 mM solution, irradiation for 30 min at ambient temperature. [b] Yields estimated from GC peak areas. [c] Molecular ions from FDMS. [d] Solution purged with argon prior to photolysis. [e] n.d. = not determined.

whereas the ketone **16** most probably arises from reaction of the corresponding triplet carbene with O_2[25] present in the experiments done under an air atmosphere. The alcohol **17** may be formed by direct insertion of the carbene into water (present in small amounts in the solvents used), but is more likely to be a photoreduction product of the ketone **16**, since (1) H_2O would have to compete with a large excess of ROH molecules (of similar reactivity toward the carbene) in the case of the alcohols and (2) purging with argon reduced both the amounts of **16** and **17** in the experiment performed in cyclohexane, whereas the water content of the solvent should not be changed markedly by this treatment.

The reduced **18** is probably also formed from the triplet carbene by double H-abstraction. The lack of formation of CH insertion products in the alcoholic solvents (also indicative of triplet carbenes[24]) can be attributed to the presence of O_2, which is known to be a powerful scavenger of triplet species.

In summary, the above results show the capability of ATEPhe to effectively insert into OH and CH bonds, proving that a highly reactive carbene (mainly in the singlet state[26]) is the intermediate in the photolysis of the diazirine.

Conclusions

4'-(1-Azi-2,2,2-trifluoroethyl)phenylalanine (ATEPhe) is a photolabile analogue of phenylalanine suitable for peptide synthesis. On illumination with wavelengths >300 nm (not destructive for most biomolecules) it rapidly eliminates N_2. The resulting carbene, predominantly present in its singlet state, inserts efficiently into OH and CH bonds. Although about 30% of the diazirine rearranges photochemically to the corresponding diazo isomer, this is not crucial, since the latter is partly (depending on the photolysis time) photolyzed to the same carbene as the parent diazirine and is not prone to acid-catalyzed decomposition, thus avoiding unspecific labeling by nonphotochemical reactions. The trifluoromethyl residue might be useful as a reporter group using ^{19}F NMR to follow the biological (noncovalent) and photochemical (covalent) binding of ligands containing ATEPhe to receptor proteins. ATEPhe should therefore prove useful in studies on the interaction of phenylalanine and biologically active peptides with other biomolecules.

Experimental Section

General Methods. UV/vis spectra were recorded on a Pye Unicam SP 8-100 spectrophotometer, ^1H NMR spectra on a Hitachi Perkin-Elmer R 600 spectrometer (60 MHz; Me_4Si as internal standard), and electron impact mass spectra on a Du Pont Type 21-492 instrument. GC analyses were run on a Perkin-Elmer gas chromatograph 990 using a Durabond DB-1 column (J&W); GC/MS and FDMS data were obtained on a Finnegan MAT 112 instrument. Column chromatography was performed on silica gel 60 (70–200 mesh), Lichroprep RP-8 (Lobar column size B), Merckogel OR-PVA 2000 (all from Merck, Darmstadt, FRG), or Sephadex LH 20 (Pharmacia, Uppsala, Sweden). Fractions were collected with an Ultrorac 7000 fraction collector equipped with an Uvicord II detector (LKB, Bromma, Sweden).

Thin-layer chromatography was performed on precoated silica gel 60 F_{254} plates (Merck, Darmstadt, FRG) using the following solvent systems: (A) petroleum ether/dichloromethane 2:1; (B) benzene/acetic acid 7:1; (C) trichloromethane/methanol/acetic acid 95:5:3; (D) trichloromethane/methanol/water 65:25:4; (E) 2-butanone/acetone/water 60:6:10; (F) 2-butanol/ethyl acetate/water 14:12:5; (G) ethyl acetate/methanol/water 5:2:1 (all v/v). Spots were visualized by fluorescence quenching under a 254-nm UV light source, by iodine vapor or with ninhydrin. Photolyses were performed as described,[7] using a Philips HPK 125 W/L high-pressure mercury lamp or in a Rayonet photochemical reactor equipped with 16 RPR 3500-Å lamps.

3-(4-Methylphenyl)-3-(trifluoromethyl)-3*H*-diazirine (1). Compound **1** was prepared essentially as described[7] with one exception: introduction of the oximino function into 2,2,2-trifluoro-1-(4-methylphenyl)-1-ethanone yielding 2,2,2-trifluoro-1-(4-methylphenyl)-1-ethanone oxime was achieved by heating the ketone with 3 equiv of hydroxylamine hydrochloride in pyridine for 4 h at 70 °C. Then the pyridine was removed in vacuo, and the residue was taken up in ethyl acetate and extracted with 0.1 N aqueous HCl. After the organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo, the residue was crystallized from *n*-hexane; yield, 92%.

3-(4-(Bromomethyl)phenyl)-3-(trifluoromethyl)-3*H*-diazirine (2). To a stirred solution of 5.0 g (24.98 mmol) of **1** in 100 mL of tetrachloromethane 4.44 g (1 equiv) of powdered NBS was added and the mixture warmed up to 70 °C. After 100 mg of AIBN was added and the temperature was gradually raised, the reaction started, apparent by vigorous boiling. After 20 min, the reaction mixture was cooled, the succinimide separated by filtration, and the filtrate concentrated in vacuo. Subsequent column chromatography on silica gel with petroleum ether/dichloromethane (20:1) yielded **2** as a colorless oil, which crystallized at −30 °C, but melted while warming up to room temperature. The last fractions of the chromatographic separation contained, besides **2**, a few higher brominated byproducts, mainly the dibromomethyl analogue; yield, 4.8 g (69%). TLC: R_f (petroleum ether/dichloromethane 20:1) = 0.70 (**1**:0.91). MS: m/z 277 + 2 (M^+ − H, 1%; eventually M^+ of the dibromo compound (358 ± 2) − Br), 250 + 2 ($M^+ - N_2$, 20%), 199 (M^+ − Br, 16%), 171 ($M^+ - Br - N_2$, 100%), 151 (70%), 102 (17%). ^1H NMR ($CDCl_3$): δ 4.42 (s, 2 H), 7.12 and 7.43 (AA'BB' system, 4 H, aromatic H).

Diethyl 2-Acetamido-2-[4-(1-azi-2,2,2-trifluoroethyl)benzyl]malonate (3). A total of 3.85 g (17.74 mmol) of diethyl acetamido malonate was added in portions to a solution of 408 mg (17.74 mmol) of sodium in 30 mL of absolute ethanol. Then 4.5 g (16.1 mmol) of **2**, dissolved in 10 mL of ethanol, was added. The mixture was heated with stirring to 65 °C for 2 h and then concentrated in vacuo, and the residual suspension (together with the precipiate) was subjected to column chromatography on silica gel with trichloromethane/ethanol (20:1) as solvent. The fractions containing **3**, contaminated with unreacted diethyl acetamidomalonate, were concentrated in vacuo and the residue was crystallized from ethyl acetate/ether/petroleum ether, yielding 3.33 g of **3**. Rechromatography of the mother liquor with the above solvent and subsequent crystallization gave another 1.53 g (mp 110–111 °C); yield, 4.86 g (73%). TLC: R_f (trichloromethane/ethanol 20:1) = 0.40 (**2**: 0.95; diethyl acetamidomalonate: 0.23); R_f(B) = 0.69; R_f(E) = 0.95. MS: m/z 415 (M^+, 3%), 386 (28%), 327 (60%), 299 (40%), 281 (25%), 243 (75%), 226 (27), 209 (35%), 197 (30%), 173 (100%), 43 (80%). ^1H NMR ($CDCl_3$): δ 1.30 (t, 3 H), 2.02 (s, 3 H), 3.70 (s, 2 H), 4.29 (q, 4 H), 6.55 (s, br, 1 H), 7.08 (m, 4 H, aromatic H).

Anal. ($C_{18}H_{20}F_3N_3O_5$) C, H, N.

2-Acetamido-2-(ethoxycarbonyl)-3-[4-(1-azi-2,2,2-trifluoroethyl)-phenyl]propionic Acid (5). **3** (2.1 g, 5.06 mmol) was dissolved in 20 mL of dioxane and stirred with 4 equiv of 2 N aqueous NaOH: the progress of the reaction was followed by using TLC (solvent E). After 1 h, most of **3** was saponified to the monoester **5**, whereas only small amounts of the dicarboxylic acid **4** were formed. Most of the dioxane was removed in vacuo; the residual alkaline aqueous phase was extracted 3 times with ethyl acetate/ether (1:1), acidified with 1 N HCl to pH 2–3, and extracted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue crystallized from ethyl acetate/petroleum ether (mp 120–121 °C); yield, 1.6 g (82%). TLC: R_f(E) = 0.30; R_f(B) = 0.13; R_f(C) = 0.15; R_f(D) = 0.76. MS: m/z 359 ($M^+ - N_2$, 1%), 315 ($M^+ - N_2 - CO_2$, 22%), 256 (100%), 228 (44%); 151 (27%), 102 (98%), 43 (72%). ^1H NMR ($CDCl_3$): δ 1.30 (t, 3 H), 2.04 (s, 3 H), 3.69 (s, 2 H), 4.30 (q, 2 H), 6.80 (br, 1 H), 7.08 (m, 4 H, aromatic H), 9.70 (1 H).

Anal. ($C_{16}H_{16}F_3N_3O_5$) C, H, N.

Decarboxylation of **5**: 200 mg (0.52 mmol) of **5** was dissolved in 10 mL of dioxane and stirred at 80 °C. TLC showed no reaction in 5 min;

(24) Tomioka, H.; Suzuki, S.; Izawa, Y. *J. Am. Chem. Soc.* **1982**, *104*, 3156–3162 and references therein. Moss, R. A., Jones, M., Eds. "Carbenes"; Wiley: New York, 1975, Vol. I, p 2.

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(26) Singlet and triplet state may be in a rapid equilibrium.[22]

after 1 h about 50% reaction had occurred, and all of **5** had disappeared in 2.5 h. Instead, two new substances were visible on TLC with the following R_f values: R_f(B) = 0.46 and 0.54; R_f(E) = 0.92 and 0.96.

2-Acetamido-2[4-(1-azi-2,2,2-trifluoroethyl)benzyl]malonic Acid (4). **3** (2.6 g, 6.26 mmol) was stirred with 12.5 mL of 2 N aqueous NaOH for 16 h and worked up as described for **5**. The residue was crystallized from ethyl acetate/petroleum ether (mp 113–114 °C); yield, 2.03 g (90%). TLC: R_f(B) = 0.02; R_f(D) = 0.45; R_f(E) = 0.12. MS: *m/z* 287 ($M^+ - N_2 - CO_2$, 16%), 228 (100%), 225 (30%), 199 (10%), 183 (9%), 176 (12%), 172 (14%), 151 (28%), 115 (20%), 74 (36%). ^{1}H NMR (Me_2SO-d_6): δ 2.00 (s, 3 H), 3.49 (s, 2 H), 7.18 (m, 4 H, aromatic H), 7.71 (br, 1–2 H). Although crystalline, the product did not give a correct elemental analysis. To obtain purer crystals recrystallization was attempted from methanol/water. But a new compound was formed in almost quantitative yield, according to TLC, identical with the decarboxylation product **6**. Saponification of **5** with 2.5 equiv of 2 N aqueous NaOH in dioxane led within 2 h to a product identical with **4**; yield from 2.0 g of **5**, 1.71 g (92%).

D,L-*N*-Acetyl-4′-(1-azi-2,2,2-trifluoroethyl)phenylalanine (6). A stirred solution of 3.0 g (8.35 mmol) of **4** in 30 mL of dioxane was warmed up in a preheated oil bath to 80 °C. TLC (solvents D and E) showed the reaction to be complete after 60 min. After cooling to room temperature, the solvent was removed in vacuo and the residue partitioned between 5% aqueous $NaHCO_3$ and ethyl acetate/ether 1:1. The aqueous phase was acidified with 1 N HCl to pH 2–3 and extracted 3 times with ethyl acetate. The combined organic extracts were washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue crystallized from ethyl acetate/petroleum ether (mp 131–132 °C); yield, 2.3 g (87%). TLC: R_f(B) = 0.10; R_f(C) = 0.17; R_f(D) = 0.73; R_f(E) = 0.33. MS: *m/z* 315 (M^+, 1%), 287 ($M^+ - N_2$, 17%), 228 (100%), 225 (28%), 205 (10%), 199 (10%), 183 (8%), 172 (14%), 151 (15%), 74 (36%), 58 and 43 (100%). ^{1}H NMR (Me_2SO-d_6): δ 1.82 (s, 3 H), 3.07 (br, 2 H), 4.50 (m, 1 H), 7.32 (m, 4 H, aromatic H) narrow AA′BB′ system), 8.20 (d, br, 1 H).

Anal. ($C_{13}H_{12}F_3N_3O_3$) C, H, N.

L-4′-(1-Azi-2,2,2-trifluoroethyl)phenylalanine (7). (a) Deacetylation of **6** with acylase I from porcine kidney (E.C. 3.5.1.14, Serva, Heidelberg, FRG). 1.0 g (3.17 mmol) of **6** was neutralized with 1 N NaOH, taken up in 100 mL of 20 mM phosphate buffer (pH 7.4) and 20 mg of $CoCl_2 \cdot 6H_2O$, and 25 mg of the enzyme (specific activity, 4000 U/mg) was added. After 24 h at 37 °C only minor amounts of a new compound giving a purple color with ninhydrin were visible on TLC (solvent E); therefore another 25 mg of acylase was added, and the reaction mixture was kept for additional 2 days at 37 °C. Thereafter, 1 N aqueous HCl was added to pH 2–3, and the mixture was extracted 3 times with ethyl acetate to remove the unreacted *N*-acetyl compound. The aqueous phase was subjected to column chromatography on a strongly acidic ion exchanger (Merck, Darmstadt, FRG) in the pyridinium form. After being washed with water, the free amino acid was eluted with 20% aqueous pyridine. The solvent was removed in vacuo, the residue was taken up in the sufficient amount of 2 N NaOH, and with acetic acid the pH was adjusted to 6.5. After addition of methanol/acetone the product crystallized on storage at 0 °C; yield, 215 mg (50%).

(b) Deacetylation with acylase from *Aspergillus* (Sigma, Munich, FRG): 1.0 g (3.17 mmol) of **6**, neutralized with 1 N NaOH, was dissolved in 80 mL of 20 mM phosphate buffer (pH 6.6; 5×10^{-4} M $CoCl_2$) and incubated at 37 °C with 80 mg of the microbial enzyme (specific activity, 66 U/mg). After 10 min, TLC showed a new ninhydrin-positive spot. After 8 h, the acidified mixture was extracted with ethyl acetate and the aqueous phase was concentrated in vacuo. The residue was dissolved in a mixture of methanol/aqueous TFA and subjected to a Lobar RP-8 column (size B; Merck, Darmstadt, FRG). Elution with 0.1% aqueous TFA/methanol 9:1 removed a brown impurity derived from the enzyme preparation. Subsequently, the free amino acid **7** was eluted with a linear gradient by changing the above solvent mixture from a 9:1 to a 1:9 ratio. The fractions containing **7** were pooled and concentrated in vacuo, and the residue was crystallized from water/methanol; yield, 325 mg (75%). TLC: R_f(E) = 0.27. MS: *m/z* 201 ($M^+ - N_2 - CO_2$, 26%), 171 (40%), 44 (100%). UV (in H_2O): λ_{max} (ε) 222 (15 000), 264 (350), 271 sh (290), 354 nm (320). $[\alpha]_D$ −15.8° (*c* 0.176, H_2O).

Anal. ($C_{11}H_{10}F_3N_3O_2$) C, H, N.

L-*N*-*tert*-Butoxycarbonyl-4′-(1-azi-2,2,2-trifluoroethyl)phenylalanine (9). Introduction of the Boc group was performed with di-*tert*-butyl pyrocarbonate in dioxane/aqueous NaOH as described[14] and the N-protected amino acid crystallized from ether/petroleum ether at −30 °C (mp 101–102 °C dec with foaming); yield, 81%. TLC: R_f(B) = 0.73; R_f(C) = 0.80; R_f(D) = 0.74. MS: *m/z* 373 (M^+, 1%), 317 (7%), 289 (35%), 246 (17%), 245 (8%), 228 (100%), 226 (17%), 172 (11%), 171 (12%), 151 (11%), 59 (18%), 57 (70%). ^{1}H NMR ($CDCl_3$): δ 1.33 (s, 9 H), 3.11 (d, 2 H), 4.52 (m, 1 H), 7.16 (m, 4 H, aromatic H), 9.06 (s, 1 H). UV (in $CHCl_3$): λ_{max} (ε) 265 (405), 272 sh (300), 358 nm (330). $[\alpha]_D$ +18.6° (*c* 3.55, $CHCl_3$).

Anal. ($C_{16}H_{18}F_3N_3O_4$) C, H, N.

L-4′-(1-Azi-2,2,2-trifluoroethyl)phenylalanine Methyl Ester (10). A total of 9.6 μL (0.11 mmol) of thionyl chloride was dissolved in 120 μL of methanol at −15 °C. Then 30 mg (0.11 mmol) of **7** was added. The resulting suspension was allowed to warm up to room temperature and stirred overnight, giving a clear solution. Concentration in vacuo yielded **10** as a white solid. TLC showed one major new spot (R_f(E) = 0.71) red-violet with ninhydrin) besides a small amount of unreacted **7**. The product was used without further purification for the synthesis of **11**.

L-*N*-*tert*-Butoxycarbonyl-β-*tert*-butylaspartyl-4′-(1-azi-2,2,2-trifluoroethyl)phenylalanine Methyl Ester (11). A total of 51 mg (0.716 mmol) of L-*N*-*tert*-butoxycarbonyl-β-*tert*-butylaspartic acid was dissolved in 2 mL of dichloromethane and neutralized with 19.2 μL (0.176 mmol) of NMM. After the mixture cooled to −15 °C, 23.3 μL (0.17 mmol) of *iso*-butyl chloroformate was added and the mixture kept for 8 min at this temperature. The white residue obtained in the preparation of **10** was dissolved in 1 mL of dichloromethane, neutralized with NMM, and then added to the above mixture, and the cooling bath was removed. After 2 h, the reaction mixture was concentrated in vacuo and separated by column chromatography on Merckogel OR-PVA 2000 with methanol as eluent; yield, 40 mg (81%), colorless oil. TLC: R_f(B) = 0.58 (**10**, 0.10; BocAsp(*t*-Bu), 0.42); R_f(C) = 0.66. MS: *m/z* 559 (M^+ + H, 1%), 428 (18%), 418 (7%), 374 (4%), 258 (6%), 244 (17%), 243 (9%), 242 (10%), 188 (32%), 174 (6%), 160 (5%), 144 (10%), 131 (30%), 87 (100%), 57 (98%).

L-Aspartyl-4′-(1-azi-2,2,2-trifluoroethyl)phenylalanine Methyl Ester (12). A total of 35 mg (0.063 mmol) of **11** was treated with 2 mL of dichloromethane/TFA 1:1 for 30 min at room temperature. The solvent was removed in vacuo and the residue subjected to gel permeation chromatography on Sephadex LH-20 with methanol as eluent. The resulting product (pure on TLC) was crystallized from water/methanol; yield, 20 mg (79%). TLC: R_f(C) = 0.05; R_f(D) = 0.54; R_f(E) = 0.16; R_f(G) = 0.66. On paper electrophoresis, the product behaves as a neutral species at pH 6.5, whereas at pH 1.9 it migrates to the cathode.

Amino acid analysis: Asp 1.00 (1), Phe[§] 0.51 (1). Phe[§] corresponds to the main hydrolysis product of ATEPhe; its retention time is identical with that of Phe. It is not clear, whether the low value is due to a correction factor different from Phe or to the formation of various side products which are not detected, or appear together with NH_4^+ from the column. It is worth mentioning that also in **13** a peak appeared with the retention time of Phe and a relative intensity of 0.51 as compared to the other amino acids.

A product identical with **12** according to TLC was obtained by reacting the *N*-hydroxysuccinimide ester of Ddz-Asp(*t*-Bu) with the free amino acid **7** (TLC of the reaction product: R_f(B) = 0.31; R_f(C) = 0.43; R_f(D) = 0.62), esterification with diazomethane (R_f(B) = 0.51; R_f(C) = 0.52; R_f(D) = 0.95) and cleavage of the protective groups as above. The product was purified on a Lobar RP-8 column with methanol/H_2O: 3/7 as eluent. **12** on TLC behaves similar as underivatized Aspartame (R_f(D) = 0.45; R_f(E) = 0.15; R_f(G) = 0.49), but as expected somewhat more lipophilic.

[4′-(1-Azi-2,2,2-trifluoroethyl)phenylalanyl[4]]-Leu-enkephalin ([ATEPhe[4]]-Leu-enkephalin) (13). (a) Boc-Gly-Gly-OEt. The dipeptide was prepared from 1.75 g (10 mmol) Boc-Gly and 1.67 g (12 mmol) of Gly-OEt·HCl by means of the mixed-anhydride method; yield, 2.5 g (96%). TLC: R_f(C) = 0.57 (brownish color with ninhydrin).

(b) Ddz-Tyr(*t*-Bu)-Gly-Gly-OH. The Boc group of the above dipeptide was removed by 50% TFA in dichloromethane. A total of 1.22 g (2.65 mmol) of Ddz-Tyr(*t*-Bu) was activated by the mixed-anhydride method and coupled to 1.0 g (3.66 mmol) of Gly-Gly-OEt TFA; yield, 1.56 g (99%, crude product). TLC: R_f(B) = 0.25; R_f(C) = 0.60; R_f(D) = 0.95. The tripeptide ethyl ester was saponified with 1.1 equiv of aqueous NaOH in dioxane; yield, 1.22 g (82%). TLC: R_f(B) = 0.05; R_f(C) = 0.14; R_f(D) = 0.47.

(c) Boc-ATEPhe-Leu-OMe. A total of 234 mg (0.627 mmol) of **9** was coupled to 181 mg (1.0 mmol) of Leu-OMe HCl by the mixed-anhydride method; yield: 283 mg (90%). The product crystallized from ethyl acetate/petroleum ether (mp 125–126 °C). TLC: R_f(B) = 0.65; R_f(C) = 0.79. MS: *m/z* 472 ($M^+ - N_2$, 7%), 416 ($M^+ - N^2 - C_4H_8$, 55%), 355 (70%), 272 (50%), 228 (60%), 227 (25%), 201 (100%), 172 (20%), 144 (18%), 141 (40%), 86 (50%), 57 (>100%).

(d) Ddz-Tyr(*t*-Bu)-Gly-Gly-ATEPhe-Leu-OMe. A total of 250 mg (0.5 mmol) of the above dipeptide was treated with 1 mL of 50% TFA in dichloromethane to remove the Boc group. The product was coupled to 423 mg (0.75 mmol) of the tripeptide from (b) by DCC/HOBt. After 16 h at room temperature the reaction mixture was concentrated in vacuo and taken up in a small amount of methanol, and the precipitated di-

cyclohexylurea was removed by filtration. Purification of the pentapeptide was achieved by gel permeation chromatography on Sephadex LH-20 with methanol as eluent. The product crystallized spontaneously in the glass tubes of the fraction collector (mp 187–189 °C dec); yield, 355 mg (75%). TLC: R_f(B) = 0.24; R_f(C) = 0.59; R_f(D) = 0.59. UV: A solution of 3.55 mg of the product in 5 mL of methanol (0.75 mM) at 359 nm showed an absorbance of 0.236; calculated from ϵ_{358} 320, A = 0.240.

(e) Tyr-Gly-Gly-ATEPhe-Leu (13). A total of 350 mg (0.37 mmol) of the protected pentapeptide was saponified with 200 μL of 2 N aqueous NaOH in 1 mL of methanol. After 1 h, the solution was neutralized with acetic acid and subjected to column chromatography on silica gel with solvent D as eluent; yield, 280 mg (81%). TLC: R_f(C) = 0.24; R_f(D) = 0.67. For removal of the residual protective groups the peptide acid was treated with 5% TFA in dichloromethane for 10 min (cleavage of the Ddz group; TLC: R_f(D) = 0.58), followed by 50% TFA in dichloromethane (cleavage of the *tert*-butyl ester; TLC: R_f(D) = 0.42). After evaporating the solvent in vacuo, the residue was purified by column chromatography on silica gel with solvent D, followed by gel permeation chromatography on Sephadex LH-20 with methanol as eluent; yield, 140 mg (83%).

Amino acid analysis: Gly 1.95 (2); Leu 1.09 (1); Tyr 1.00 (1); Phe[§] 0.51 (1) ([§]compare **12**).

The purity of the product was checked by HPLC (column, RP-8, 5 μm, 250 × 4 mm; solvent, linear gradient from 30% to 50% aqueous acetonitrile containing 0.1% TFA; gradient time, 10 min; flow rate, 1 mL/min; detection, UV at 277 and 354 nm; retention time, 6.0 min). Separation on a preparative scale could not be achieved by this gradient due to the limited solubility of the peptide in this solvent but using a 60–80% gradient of aqueous methanol containing 0.1% TFA.

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Registry No. 1, 87736-85-4; **2**, 92367-11-8; **3**, 92367-12-9; **4**, 92367-13-0; **5**, 92367-14-1; **6**, 92367-15-2; **7**, 92367-16-3; **8**, 92396-67-3; **9**, 92367-17-4; **10**, 92367-18-5; **11**, 92367-19-6; **12**, 92367-20-9; **13**, 92367-21-0; **14**, 92367-22-1; **15**, 92367-23-2; **16**, 92367-24-3; **17**, 92367-25-4; **18**, 92367-26-5; **19**, 92396-68-4; **20**, 92396-69-5; Boc-Gly-OH, 4530-20-5; H-Gly-OEt·HCl, 623-33-6; Boc-Gly-Gly-OEt, 25438-03-3; H-Gly-Gly-OEt, 627-74-7; Ddz-Tyr(Bu-*t*)-OH, 70266-05-6; Ddz-Tyr(Bu-*t*)-Gly-Gly-OEt, 92367-33-4; Boc-ATEPhe-Leu-OMe, 92367-31-2; Ddz-Tyr(Bu-*t*)-Gly-Gly-OH, 92367-30-1; H-ATEPhe-Leu-OMe, 92367-34-5; H-Leu-OMe·HCl, 7517-19-3; Ddz-Tyr(Bu-*t*)-Gly-Gly-ATEPhe-Leu-OMe, 92367-32-3; Ddz-Tyr(Bu-*t*)-Gly-Gly-ATEPhe-Leu-OH, 92367-35-6; Ddz-Asp(*t*-Bu)-OSu, 92367-27-6; Ddz-Asp(*t*-Bu)-ATEPhe-OH, 92367-28-7; Ddz-Asp(*t*-Bu)-ATEPhe-OMe, 92367-29-8; 2,2,2-trifluoro-1-(4-methylphenyl)-1-ethanone, 394-59-2; 2,2,2-trifluoro-1-(4-methylphenyl)-1-ethanone oxime, 75703-25-2; sodium diethyl acetamide malonate, 41679-89-4; L-*N*-*tert*-butoxycarbonyl-β-*tert*-butyl-aspartic acid, 1676-90-0.

Supplementary Material Available: Scheme and experimental details of the synthesis of D,L-*N*-acetyl-4′-(trifluoroacetyl)-phenylalanine and its oxime (7 pages). Ordering information is given on any current masthead page.

Circular and Flip-Flop Hydrogen Bonding in β-Cyclodextrin Undecahydrate: A Neutron Diffraction Study†

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Abstract: β-Cyclodextrin crystallizes from water in monoclinic space group $P2_1$ with a = 21.261 (6) Å, b = 10.306 (3) Å, c = 15.123 (4) Å, β = 112.3 (5)°, V = 3065.8 Å^3, Z = 2, D_x = 1.462 g/cm^3, FW = 1351.2, $(C_7H_{10}O_5)_7(11 \pm 0.5)H_2O$ per asymmetric unit. The crystal structure was initially determined from X-ray data, and H atoms were located from a 6610 neutron F_s' above σ, measured at 293 K, diffractometer equipped with a BF_3 counter, Be monochromator, λ = 1.015 Å, flux 10^6 n cm^{-2}·s^{-1}, 0.56 Å resolution, full-matrix least-squares refinement, R = 6.8%. There are 53 H bonds per asymmetric unit of which 35 are normal O—H···O and 18 are of the flip-flop type O—H···H—O, representing a statistical average O—H···O ⇌ O···H—O. The 11 water molecules are distributed over 16 positions, 8 in the cavity of β-CD (6.13 water molecules) and 8 in interstices (4.88 water molecules). Cavity waters form only 2 H bonds to enclosing β-CD and 6 contacts to neighboring β-CD. Their positions are better occupied and they are better ordered than "outside" water in interstices, an effect probably due to the hydrophobic character of the cavity. Flip-flop O—H···H—O bonds are interconnected to produce more extended systems, with one infinite flip-flop chain running through the whole crystal structure. Since the two states are energetically near-equivalent, flip-flop H bonds are entropically favored. Flip-flop bonds are also observed between all seven intramolecular, interglucose O(2), O(3) hydroxyls, O(2)—H···H—O(3), and explain the unusual conformational stiffness of β-CD in solution, compared with the more flexible α-CD which does not exhibit such flip-flop H bonds in the crystalline state. As in the α-CD·6H_2O crystal structure, β-CD·11H_2O contains some circularly closed, H-bonded systems which also involve flip-flops. Circles combined with flip-flop can form an entropically favorable system which could occur in water and in the hydration of macromolecules, i.e., β-CD·11H_2O can be considered as the frozen state of hydrated β-CD.

When starch is degraded by a special type of amylases called glucanotransferases, a family of cyclically closed oligosaccharides is obtained. They are composed of six to eight α(1–4) linked glucoses and called α-, β-, γ-cyclodextrins (α-CD, β-CD, γ-CD). Due to their annular structure, they are able to form inclusion complexes with a great variety of substrate (guest) molecules[1-5] under the proviso that the substrates are small enough to fit physically into the central cavities of the cyclodextrin rings.[1,2]

† Topography of Cyclodextrin Inclusion Complexes, Part 20. For Part 19 see: Betzel, Ch., et al. *J. Incl. Phenom.* **1983**, *1*, 181–191. For Part 18 see: Lindner, K.; Saenger, W. *Carbohydr. Res.* **1982** *107*, 7–16.

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