unusual stability of these enzyme–inhibitor complexes be due to a simple lowering of the microscopic dielectric constant at the active site, as compared with bulk solution?. The present results indicate that such effects on equilibria of binding should be negligible for hemiacetal formation and markedly adverse for thiohemiacetal formation. The observed strength of binding, all the more remarkable in view of these observations, can therefore be ascribed to specific forces of attraction that are present at the active site.

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Registry No. CH_3CHO, 75-07-0; NH_3, 7664-41-7; CH_3OH, 67-56-1; CH_3SH, 74-93-1; CH_3NO_2, 75-52-5; CH_3NH_2, 74-89-5; $HOCH_2CH_2OH$, 107-21-1; $CH_3CH(OH)_2$, 4433-56-1; $CH_3CH(OH)OCH_3$, 563-64-4; $CH_3CH(OCH_3)_2$, 534-15-6; $CH_3CH(OH)SCH_3$, 84418-46-2; $CH_3CH(OH)CH_2NO_2$, 3156-73-8; $CH_3CH(OCH_2-)_2$, 497-26-7; $HOCH_2CH_2NH_2$, 141-43-5; $CH_3CH{=}NCH_3$, 14777-29-8; $HOCH_2CH_2CH_2OH$, 504-63-2; $HOCH_2CH_2NH_3^+$, 22852-66-0.

Solution Forms of Bouvardin and Relatives from NMR Studies. 6-*O*-Methylbouvardin

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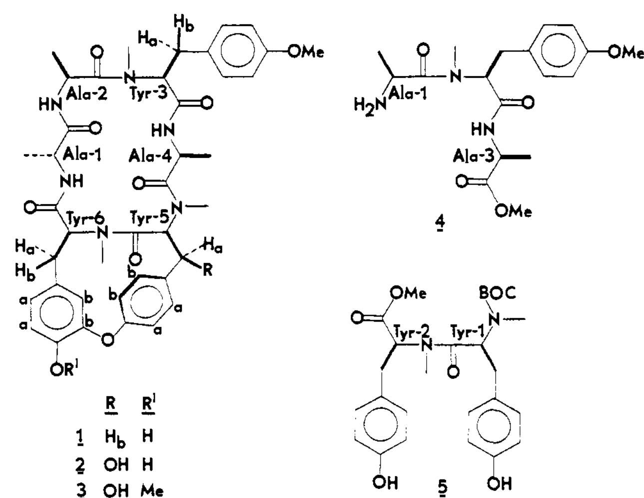
Abstract: ^{1}H and ^{13}C NMR studies indicate that the predominant stereoisomer and conformer in solution for the potent natural antitumor agents deoxybouvardin (**1**), bouvardin (**2**), and the newly isolated and equally active 6-*O*-methylbouvardin (**3**) is that found in the solid state by X-ray diffraction. Unusual features in the spectra, all in the vicinity of the 14-membered ring, include an aromatic proton absorbing unusually far upfield at δ 4.35, a vicinal H–C–O–H coupling constant of 10.2 Hz, aryl carbons ortho to an ether oxygen absorbing at δ 124.2–125.9, and a geminal coupling constant of −20 Hz between the methylene protons in a tyrosine residue. A minor stereoisomer (~15%) separated by a 20.6 kcal/mol barrier is observed for **1**–**3**; variable-temperature ^{1}H NMR studies on model *N*-methyl peptides indicate this stereoisomer to differ in rotation about the Tyr-5 and/or Tyr-3 amide bond. Since the antitumor activities of six compounds differing in substitution on Tyr-5 and Tyr-6 do not vary appreciably while a change in Tyr-3 results in loss of activity, the rigid 14-membered ring portion of the molecule is not the active part but serves to get the rest of the molecule into the active conformation.

Deoxybouvardin (**1**) and bouvardin (**2**) are natural cyclic hexapeptides, possessing strong antitumor activity, and constituted



from two L-alanines, a D-alanine, and three modified *N*-methyl-L-tyrosines.[2] Their most unusual structural feature is a 14-membered ring formed by oxidative coupling of the phenolic rings of two adjacent tyrosine units; this ring contains meta- and para-disubstituted benzene rings and a *cis*-peptide grouping. An X-ray study on **2** and spectral comparison of **1** and **2** gave their structures, but due to the complexity of their NMR spectra, very few of their NMR parameters were assigned. Analytical HPLC indicated the presence of two stereoisomers in chloroform solution,[3] but efforts to separate them on a preparative scale failed since they equilibrate at room temperature.

We report (a) the isolation of 6-*O*-methylbouvardin (**3**), an active minor component of *Bouvardia ternifolia* obtained during isolation of large quantities of **1** and **2** for biological testing, (b) ^{1}H and ^{13}C NMR studies on **1**–**3** which provide evidence on the shapes of the two major species which are observed in chloroform solution for each of these substances, and (c) structure–activity results which indicate how various portions of the molecule are involved in the activity.

Results and Discussion

6-*O*-Methylbouvardin was assigned structure **3** by comparison of its ^{1}H (Table I) and ^{13}C (Table II) NMR spectral parameters with those of **1** and **2** (see Figure 1 for ^{1}H NMR spectrum of **2**). The structure was confirmed by converting **2** to **3** with diazomethane.

The ^{1}H and ^{13}C shift assignments in Tables I and II for bouvardin (**2**) are consistent with extensive ^{1}H–^{1}H and ^{1}H–^{13}C decoupling results; the resonances of all carbons bearing hydrogens were umabiguously correlated with the resonances of those hydrogens. The assignment problems were thus reduced to which set of δ's and *J*'s belonged to which alanine, which *N*-Me ^{13}C and

(1) (a) Department of Chemistry. (b) College of Pharmacy.
(2) Jolad, S. D.; Hoffmann, J. J.; Torrance, S. J.; Wiedhopf, R. M.; Cole, J. R.; Arora, S. K.; Bates, R. B.; Gargiulo, R. L.; Kriek, G. R. *J. Am. Chem. Soc.* **1977**, *99*, 8040.
(3) Hoffmann, J. J.; Torrance, S. J.; Cole, J. R. *J. Chromatogr. Sci.* **1978**, *17*, 287. The barrier between these forms is about 20 kcal/mol, which is the approximate boundary between configurations and conformations. We have found it more convenient to use the former terminology for them.

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Table I. ^{1}H NMR Shifts (δ) and Coupling Constants (Hz, in Parentheses) for the Major Stereoisomers of 1–3 and Temperature Coefficients of Shifts for 2 (ppm/10^3 °C) in $CDCl_3$

	1	2	3	temp coeff. for 2[a]
Ala-4β	1.09 d (6.7)	1.08 (6.6)	1.10 (6.7)	0.8
Ala-2β	1.31 d (7.3)	1.30 (6.8)	1.30 (7.3)	−0.4
Ala-1β	1.36 d (7.3)	1.37 (6.9)	1.37 (6.7)	−0.8
Tyr-5β$_a$	2.62 dd (11.4, 2.4)	5.08 (10.2, 1.8)	5.09 bs	−0.8
Tyr-6 N-Me	2.70 s	2.74	2.75	−0.3
Tyr-3 N-Me	2.87 s	2.87	2.88	0.2
Tyr-6β$_a$	2.91 dd (20.2, 4.6)	2.91 (18.8, 3)	2.91 (18.8, 3)	0.0
Tyr-6β$_b$	3.00 dd (20.2, 12.4)	3.12 (18.8, 10.8)	3.14 (18.8, 11.6)	−0.6
Tyr-5 N-Me	3.11 s	3.33	3.34	−0.3
Tyr-3β	3.33 m	3.35	3.35	0.2
Tyr-3α	3.61 dd (9.8, 6.1)	3.62 (9.2, 5.9)	3.62 (9.8, 6.1)	−0.8
Tyr-5β$_b$	3.65 dd (11.4, 8.6)			
Tyr-3 O-Me	3.80 s	3.80	3.80	−0.5
Tyr-6 O-Me			3.95 s	
Tyr-6δ$_b$	4.35 bs	4.35 d (1.8)	4.35 bs	1.9
Ala-2α	4.42 ~p (7)	4.38 (7)	4.40 (7)	−0.8
Tyr-6α	4.56 dd (12.4, 4.6)	4.37 m	4.38 dd (11.6, 3)	−0.6
Ala-1α	4.79 ~p (7)	4.76 (7)	4.77 (7)	0.3
Ala-4α	4.81 ~p (7)	4.90 (7)	4.90 (7)	−0.4
Tyr-5α	5.42 dd (8.6, 2.4)	5.36 d (1.8)	5.39 bs	−0.4
Ala-4 NH	6.48 d (7)	6.71 (7.7)	6.70 (7.9)	−1.4
Tyr-6δ$_a$	6.51 dd (8.5, 1.8)	6.51 (8.1, 1.8)	6.59 ~d (8.5)	−0.6
Ala-2 NH	6.52 d (7.3)	6.54 (7.1)	6.51 (6.7)	−6.8
Tyr-6 OH	6.53 bs	6.46 s		−9.2
Tyr-5 OH		6.54 d (10.2)	6.50 bs	−2.8
Ala-1 NH	6.76 d (7.9)	6.77 (7.9)	6.75 (7.9)	−0.7
Tyr-6ε$_a$	6.81 d (8.5)	6.81 (8.1)	6.82 (8.5)	−0.4
Tyr-3ε	6.83 ~d (8.5)	6.84 (8.7)	6.84 (8.5)	−0.6
Tyr-5ε$_b$	6.83 dd (8.5, 2.4)	6.95 (8.7, 2.2)	7.01 (8.5, 2.2)	−0.5
Tyr-3δ	7.05 ~d (8.5)	7.05 (8.7)	7.05 (8.5)	−0.3
Tyr-5ε$_a$	7.20 dd (8.5, 2.4)	7.23 (8.5, 2.2)	7.26 (8.5, 2.2)	−0.8
Tyr-5δ$_b$	7.27 dd (8.5, 1.8)	7.50 (8.7, 2.2)	7.51 (8.5, 2.4)	−0.3
Tyr-5δ$_a$	7.43 dd (8.5, 1.8)	7.38 (8.5, 2.2)	7.39 (8.5, 2.4)	−0.6

[a] Positive values correspond to downfield shifts with increasing temperature; the range studied was −55 to +45 °C.

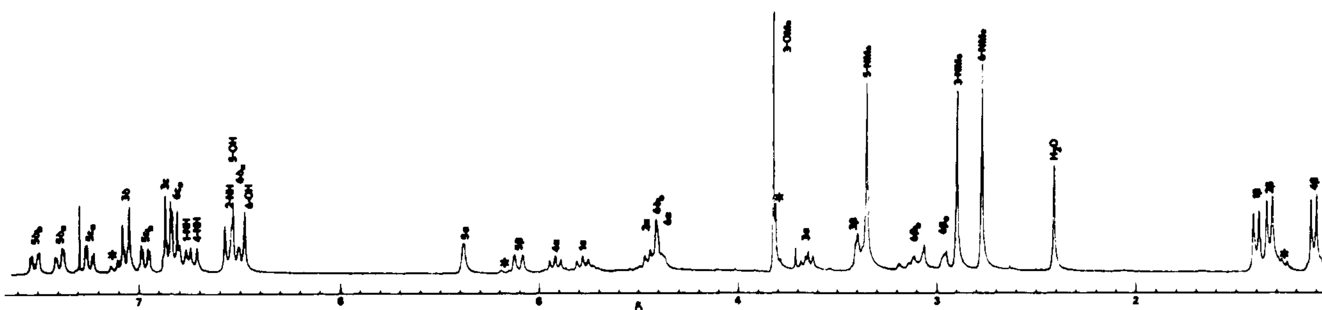

Figure 1. 250-MHz ^{1}H NMR spectrum of bouvardin (2); asterisks mark peaks from 15% stereoisomer.

^{1}H shifts belonged to which tyrosine, etc. How these assignments were made is described below. Complete assignments were made for the major stereoisomers of **1–3**, but as the evidence regarding which alanine is which and which carbonyl is which was not conclusive, these assignments must be regarded as tentative. Some of the assignments are based on the assumption that the major conformer in solution is the same as that observed in the X-ray study on bouvardin (**2**). This is certainly true for the rigid Tyr-5 and Tyr-6 portion of the molecule, and support will be presented later for the view that it is at least approximately true for the rest of the molecule.

Tyrosine α-Methinyls and β-Methylenes. The peaks due to Tyr-5 were umambiguously assignable, as only this tyrosine in **2** has a β-methinyl rather than a β-methylene group. The 10.2-Hz coupling constant observed between the Tyr-5β$_a$ and attached OH protons in **2** supports a dihedral angle close to 180° between these protons and thus an intramolecular H bond to the Tyr-5 carbonyl group as proposed earlier.[2] The coupling constants of 2 Hz between the Tyr-5α and Tyr-5β$_a$ protons and 8.6 Hz between the Tyr-5α and Tyr-5β$_b$ protons are consistent with dihedral angles of 68° and 172°, respectively, expected from the crystal structure of **2**.[4,5] The Tyr-5β$_b$ proton absorbs considerably downfield from the Tyr-5β$_a$ proton because the former is deshielded by the Tyr-5 carbonyl group and because it is more in the plane of the Tyr-5 aromatic ring.

The assignments for Tyr-3 vs. Tyr-6 were initially made on the basis that the vicinal coupling constants between the Tyr-6α and Tyr-6β protons should match their X-ray dihedral angles of 65° and 175° whereas those between the Tyr-3α and Tyr-3β protons should match angles of 50° and 170°. Strong support came from the unusually negative geminal coupling constant (−20 Hz) between the Tyr-6 methylene protons (−13.8 Hz is typical);[6] this is a result of (a) an unusually small H–C_6^{β}–H angle which accompanies the unusually large C_6^{α}–C_6^{β}–C_6^{γ} angle observed[2] (118.7 (5)°; the C_3^{α}–C_3^{β}–C_3^{γ} angle of 110.6 (8)° is close to the typical value of 111.4 (5)°),[6] and/or (b) the twisting of the phenyl ring relative to the methylene protons toward the conformation with the most negative coupling constant.[7] These assignments are also

(4) Although hydrogens were not found in the bouvardin (**2**) X-ray study,[2] their positions were readily calculated from the positions of the other atoms.

(5) Pachler, K. G. R. *Spectrochim. Acta* **1964**, *20*, 581.

(6) Yang, C.-H.; Brown, J. N.; Kopple, K. D. *J. Am. Chem. Soc.* **1981**, *103*, 1715.

Table II. ^{13}C NMR Shifts (δ) for the Major Stereoisomers of 1-3 in $CDCl_3$

	1	2	3
Ala-2β	16.6 q	16.4	16.3
Ala-4β	18.5 q	18.5	18.5
Ala-1β	20.7 q	20.5	20.5
Tyr-6 *N*-Me	29.4 q	29.2	29.2
Tyr-5 *N*-Me	30.6 q	33.0	33.1
Tyr-3β	32.7 t	32.7	32.8
Tyr-6β	35.7 t	35.9	35.8
Tyr-5β	36.9 t	78.5 d	78.5 d
Tyr-3 *N*-Me	39.8 q	39.7	39.8
Ala-1α	44.6 d	44.9	44.9
Ala-4α	46.5 d	46.5	46.6
Ala-2α	47.9 d	47.9	47.9
Tyr-5α	54.4 d	53.9	54.0
Tyr-3 *O*-Me	55.3 q	55.3	55.3
Tyr-6 *O*-Me			56.3 q
Tyr-6α	57.5 d	57.8	57.7
Tyr-3α	68.4 d	68.2	68.2
Tyr-6δ_a	113.1 d	113.0	112.7
Tyr-3ϵ	114.1 d	114.1	114.1
Tyr-6ϵ_a	115.9 d	116.1	113.4
Tyr-6δ_a	121.7 d	121.7	121.2
Tyr-5ϵ_b	124.2 d	124.2	124.3
Tyr-5ϵ_a	125.9 d	125.7	125.8
Tyr-6γ	127.7 s	127.2	127.9
Tyr-3δ	130.3 d	130.3	130.4
Tyr-3γ	130.8 s	130.7	130.8
Tyr-5δ_a	131.1 d	126.9	126.9
Tyr-5δ_b	133.0 d	128.5	128.4
Tyr-5γ	135.7 s	139.8	139.5
Tyr-6ζ	143.1 s	143.2	146.8
Tyr-6ϵ_b	151.2 s	151.0	153.2
Tyr-5ζ	158.0 s	158.9	159.1
Tyr-3ζ	158.5 s	158.4	158.5
Tyr-6 CO	168.1 s	168.3	168.3
Tyr-5 CO	169.2 s	170.0	170.0
Tyr-3 CO	170.7 s	170.8	170.9
Ala-4 CO	171.8 s	172.2	172.3
Ala-1 CO	172.3 s	172.2	172.3
Ala-2 CO	172.7 s	172.5	172.6

supported by the observed changes in chemical shifts of the Tyr-6α and Tyr-6β_b protons between **1** and **2**: these protons are much closer than the corresponding Tyr-3 protons to the point of difference between **1** and **2**.

Tyrosine Aromatics. The most striking feature in the aromatic resonances is the Tyr-6δ_b proton absorption, which at δ 4.4 is over 2 ppm upfield of the other aromatic proton absorptions. This occurs because this proton lies over the Tyr-5 aromatic ring at very close range, and is precedented in such compounds as [2.2]metacyclophane.[8] The Tyr-6δ_b *carbon* is similarly the highest field aromatic carbon, but as its distance from the Tyr-5 aromatic ring is much greater, its upfield shift is not so striking. The Tyr-6δ_a and Tyr-6ϵ_a proton and carbon absorptions were readily located through decoupling experiments.

Tyr-6γ, Tyr-6ϵ_b, and Tyr-6ζ were assigned to the three carbon singlets (off-resonance) which vary most between **2** and **3**, which went with which was decided by calculation.[9] The finding that the 6ϵ_a-carbon absorbs *upfield* in 6-*O*-methylbouvardin (**3**) while 6ϵ_b absorbs *downfield* is interpretable as a γ effect[10] and strongly suggests that the methoxyl group in **3** is oriented toward 6ϵ_a, as expected on steric grounds.[11]

The 3δ and 3ϵ proton and carbon absorptions were easily identified by their doubled size. The 3γ- and 3ζ-carbons were assigned to the only two aromatic singlet absorptions which do not change among **1**–**3**, with the downfield absorption clearly belonging to the aromatic carbon bearing oxygen. These assignments are strongly supported by their very close correspondence to the analogous shifts in tripeptide **4**.

Once one of the 5δ and 5ϵ NMR absorptions was assigned, the others could be confidently related to it by decoupling experiments. The initial assignment was made on the basis that the 5ϵ protons and carbons, being ortho to oxygen, should be upfield from the 5δ protons and carbons and the 5ϵ_b proton should absorb upfield from the 5ϵ_a since the Tyr-6 ring shields the former deshields the latter. Support for the resulting assignments comes from the relatively small changes in 5ϵ ^1H and ^{13}C shifts between **1** and **2**, compared to the larger changes in 5δ shifts (δ's are much closer to the point of structural difference between **1** and **2**); also, the 5δ-carbons absorb considerably farther upfield in **2** and **3** than in **1** due to γ effects.[10]

The 5γ-carbon absorbs 4 ppm farther downfield in **2** and **3** than in **1** due to a β effect.[10] The last downfield aromatic singlet, which varies significantly among all three compounds, was assigned to 5ζ; it is downfield in **2** and **3** due to the electron-withdrawing effect of the added hydroxyl.

Tyrosine *N*-Methyls. Tyr-3 *N*-Me was assigned from its unchanging shifts among **1**–**3**. Tyr-5 *N*-Me, which is very close to the alcoholic oxygen of **2** in crystalline **2**,[2] is presumed to be the *N*-Me which undergoes large ^1H and ^{13}C shifts when this oxygen is removed. Tyr-6 *N*-Me then becomes the one which shows a slight ^1H shift between **1** and **2**; it is reasonable that the protons of this methyl should absorb upfield as they should be shielded by the Tyr-6 aromatic ring.

Alanines. Ala-4 was assigned on the basis that it is the only alanine close enough to the point of difference between **1** and **2** to have observably different chemical shifts (α-H and *N*-H). This assignment is supported by its methyl protons absorbing much farther upfield than the other alanine methyl protons, as the Ala-4-Me protons should be shielded by the 2-, 3-, and 6-carbonyl groups.

The assignments for the other two alanines were made on the basis of the temperature dependence of the NH shifts (Table I). The NH which varies in position greatly with temperature is presumably in Ala-2, because this NH cannot intramolecularly H-bond without a gross change in the conformation; on the other hand, the Ala-1 and Ala-4 NH protons can H-bond transannularly to the Ala-4 and Ala-1 carbonyl oxygens, respectively, with only slight changes from the X-ray conformation.

Carbonyls. The only two whose shifts vary significantly between **1** and **2** were taken to be in Ala-4 and Tyr-5; of these two, the 5-carbonyl was assumed to vary more and to absorb farther upfield because it has three more γ substituents.[12] Of the other four, the farthest downfield was calculated to be in Ala-2 (one more β substituent than the other three, and three γ's), the next in Ala-1 (three γ's), the next in Tyr-3 (five γ's but both close to trans), and the last in Tyr-6 (five γ's including an additional one *not* close to trans).

Major and Minor Stereoisomers. In all cases of room-temperature NMR spectra of **1**–**3**, some small peaks (asterisked in Figure 1) were observed in addition to the peaks due to the major stereoisomer. That these were due to a minor (~15%) stereoisomer separated from the major one by a barrier of ~20 kcal/mol was suggested by earlier results with analytical HPLC[3] and verified by a low-temperature ^1H NMR study on bouvardin (**2**): Crystals of **2** obtained by evaporation of a methanol solution as in the X-ray study[2] were dissolved in $CDCl_3$ at −55 °C and the spectrum was measured at 10° intervals as the temperature was raised. The small peaks did not appear until the 15 °C spectrum, and from the estimated half-life of 8 min at 15 °C for the conversion of the major stereoisomer to the minor one, the barrier was calcu-

(7) Sternhell, S. *Q. Rev., Chem. Soc.* **1969**, *23*, 236.
(8) Lindsay, W.; Stokes, P.; Humber, L.; Boekelheide, V. *J. Am. Chem. Soc.* **1961**, *83*, 943.
(9) Bates, R. B.; Beavers, W. A. "Carbon-13 NMR Spectral Problems"; Humana Press: Clifton, NJ, 1981.
(10) Clerc, J. T.; Pretsch, E.; Sternhell, S. "^{13}C-Kernresonanzspektroskopie"; Akademie Verlagsgesellschaft: Frankfurt, 1973.
(11) Arora, S. K.; Bates, R. B.; Grady, R. A.; Germain, G.; Declercq, J. P. *J. Org. Chem.* **1975**, *40*, 28.
(12) The γ effect for amide carbonyl carbons is about −3.2 ppm for each γ-carbon, with additional increments of −4.0 if fixed cis and +2.5 if fixed trans; the β effect is +2.6 ppm.[13]
(13) Johnson, C. S., Jr. *Adv. Magn. Reson.* **1965**, *1*, 33.

lated[14] to be 20.6 kcal/mol. This is reminiscent of the 20.5 kcal/mol barrier in *N,N*-dimethylformamide[14,15] and suggests that *the major stereoisomer of 2 in solution has the same trans-Tyr-3, trans-Tyr-5, cis-Tyr-6 configuration as in the crystals, and the minor stereoisomer differs in configuration at one (possibly more) of the N-methyltyrosines.*[16] It is very unlikely that the *cis*-Tyr-6 bond would change since that would put a *trans*-amide bond in the strained 14-membered ring. The visible ^{1}H and ^{13}C NMR peaks of the minor stereoisomer show differences from the major one in both the Tyr-3 and Tyr-5 absorptions, with the largest ^{1}H difference being for Tyr-5β_a. As a 180° change in rotation about *either* the Tyr-3 or Tyr-5 *N*-methyl peptide bond would probably affect the shifts of atoms in both amino acid units, it is not clear about which one (possibly both?) rotation has occurred.

Major Conformation in Solution. The ^{1}H and ^{13}C NMR absorptions of **1–3** for Ala-1, Ala-2, and Tyr-3, all in the flexible "upper" portion of the molecule, are almost identical, indicating that they share the same conformation(s) in solution. The low-temperature study described in the previous section shows that the *configurations* about the *N*-methyl-Tyr bonds are *trans*-Tyr-3, *trans*-Tyr-5, *cis*-Tyr-6 in the major solution form; the Ala N*H*–C*H* coupling constants of 6.7–7.9 Hz and Tyr-3α-H-Tyr-3β-H coupling constants are consistent with the view that *the main conformation of bovardin 2 in solution is essentially the same same as that in the crystal*, where N*H*–C*H* torsion angles of 166° (Ala-1), 157° (Ala-2), and 144° (Ala-4) and Tyr-3α-H and Tyr-3β-H torsion angles of 48° and 168° were observed.[2,4,17,18]

Table I includes the temperature coefficients of the ^{1}H NMR shifts of bouvardin (**2**) in $CDCl_3$, determined over most of the liquid range of this solvent. The Tyr-6-OH shift varies more with temperature than the Tyr-5-OH shift, indicating the former to be more involved in intermolecular H bonding.[18] The CH shifts do not vary much except for that of the Tyr-6δ_b proton, which absorbs 0.19 ppm farther downfield at the highest temperature than at the lowest. This is presumed to result from its average position being farther from the Tyr-5 aromatic ring when it is vibrating more at higher temperatures.

Mechanism of Action. Bouvardin (**2**) has been shown to inhibit protein biosynthesis.[19] In view of the very similar activities of **1–3** and other compounds[20] which differ in the groupings on the conformationally rigid Tyr-5–Tyr-6 portion of the molecule and the lack of activity of *O*-desmethylbouvardin,[21] it appears that *the inert rigid lower portion of the molecule merely serves to hold the active upper portion in the right shape for activity.* The right shape may be the predominant shape described herein, or perhaps a somewhat less thermodynamically favored conformer or even stereoisomer.

Experimental Section

250-MHz ^{1}H and 62.9-MHz ^{13}C NMR spectra were recorded on a Bruker WM-250 spectrometer, using Me_4Si as an internal standard. The ^{13}C spectra were run with both broad-band and off-resonance decoupling. Melting points were determined on a Kofler hot-stage apparatus and are uncorrected, and optical rotations were measured on a Perkin-Elmer 241 MC automatic polarimeter. HPLC separations were carried out on a Spectra-Physics Model 3500 B instrument.

Isolation of Methylbouvardin (3). Deoxybouvardin (**1**) and bouvardin (**2**) were separated from each other on a large scale by column chromatography [SiO$_2$-60, Merck: (**2** + **1**), ≥50:1, w/w] eluting with the solvent mixture Et_2O: EtOAc:MeOH (15:35:2, v/v/v).[2] Bouvardin (**2**) purified in this way was recrystallized from MeOH. However, HPLC examination (ODS reverse-phase column; mobile phase = MeOH:H_2O, 1:1, v/v; 2 mL/min; detector at 254 nm) revealed the presence of an impurity (ca. 6%). The latter (**3**) was separated from **2** by column chromatography [SiO$_2$-60, Merck: (**2** + **3**), ≥50:1, w/w] eluting with the solvent system hexane:CH_2Cl_2:MeOH (20:27:3, v/v/v). The faster-eluting material (**3**) was recrystallized from MeOH, providing colorless plates: mp 244–247 °C; $[\alpha]_D^{24}$–191° (*c* 1.0, $CHCl_3$); NMR data in Tables I and II; PS activity 134% test/control at 1 mg/kg.

Methylbouvardin (3) from Bouvardin (2). Bouvardin (**2**, 186 mg) in Et_2O:MeOH (1:1, v/v, 20 mL) was treated in the usual manner[22] with diazomethane, generated from *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich, 1 g) in Et_2O (10 mL). After 24 h at room temperature the usual workup afforded methylbouvardin (**3**, 185 mg) which was recrystallized from MeOH to colorless plates, mp 244–246 °C, $[\alpha]_D^{24}$–190° (*c* 1.0, $CHCl_3$). The melting point of an intimate mixture of natural and synthetic samples of **3** was undepressed.

NMR Absorptions of Minor Stereoisomers of 1–3. Many of the NMR peaks of the 15% stereoisomers overlap with peaks of the 85% stereoisomers. The most useful ones which did not overlap were the Tyr-3δ doublets at δ 7.11 ~d (8 Hz) in **1–3**; these peaks are due to *two* protons and thus are relatively easily observed. Other proton peaks used in **2** were for Tyr-3-OMe at δ 3.79 s, Ala-2β at δ 1.21 d (7 Hz), and Tyr-5β_a at δ 5.16 d (10 Hz). ^{13}C NMR peaks which were used were the doubled peaks for Tyr-3δ at δ 130.0 and Tyr-3ϵ at δ 114.4 and the single peak for Tyr-3ζ at δ 158.7.

Variable-Temperature Studies. Bouvardin (**2**) crystallized from methanol was dissolved in $CDCl_3$ at –55 °C and the ^{1}H NMR spectrum was measured at 10° intervals up to 45 °C. The resulting temperature coefficients of the ^{1}H shifts of the major stereoisomer are given in Table I. The minor stereoisomer appeared at 15 °C with an estimated half-life of 8 min, indicating a barrier of 20.6 kcal/mol.[14]

A barrier of 17.5 kcal/mol was measured for tripeptide **4**[23] in two ways: (a) As with bouvardin (**2**), crystals of the type used in an X-ray study[21] (in this case revealing a *cis*-*N*-methyl-Tyr amide bond) were dissolved in $CDCl_3$ at –50 °C and the ^{1}H NMR spectrum was recorded as the temperature was raised. The half-life for appearance of the trans isomer (50% at equilibrium) was 5 min at –23 °C. (b) The temperature of a nitrobenzene solution of **4** was raised until coalescence was observed for various groupings, i.e., *N*-methyls, T_c = 48 °C, $\Delta\nu$ = 2.7 Hz; C-methyls, T_c = 55 °C, $\Delta\nu$ = 6.5 Hz. Room temperature values for the *cis* isomer: ^{1}H NMR δ 1.06 d (6.2, Ala-1β), 1.35 d (7.4, Ala-3β), 1.67 s (NH_2), 2.88 s (NMe), 2.96 m and 3.17 m (Tyr-β), 3.20 m (Ala-1α), 3.73 s (CO_2Me), 3.77 s (ArO*Me*), 4.51 m (Ala-3α), 4.71 dd (11.4, 4.8, Tyr-α), 6.80 d (8.5, Tyr-ϵ), 7.04 d (8.5, Tyr-δ), 8.83 d (7.9, NH); ^{13}C NMR, δ 18.2 (Ala-3β), 21.9 (Ala-1β), 28.7 (NMe), 32.7 (Tyr-β), 46.9 (Ala-1α), 48.0 (Ala-3α), 52.5 (CO_2*Me*), 55.3 (ArO*Me*), 61.6 (Tyr-α), 114.2 (Tyr-ϵ), 130.0 (Tyr-δ), 158.6 (Tyr-ζ), 169.4 (Tyr-CO), 173.6 (CO_2Me), 177.6 (Ala-3-CO). For the trans isomer: ^{1}H NMR δ 1.20 d (7.0, Ala-1β), 1.34 d (7.4, Ala-3β), 1.67 s (NH_2), 2.92 s (NMe), 3.10 m and 3.22 m (Tyr-β), 3.67 m (Ala-1α), 3.73 s (CO_2Me), 3.78 s (ArO*Me*), 4.55 m (Ala-3α), 5.30 dd (11.4, 7.1, Tyr-α), 6.66 d (7.9, NH), 6.81 d (8.5, Tyr-ϵ), 7.13d (8.5, Tyr-δ); ^{13}C NMR δ 18.2 (Ala-3β), 20.7 (Ala-1β), 31.0 (NMe), 32.7 (Tyr-β), 47.2 (Ala-1α), 48.1 (Ala-3α), 52.4 (CO_2*Me*), 55.3 (ArO*Me*), 58.0 (Tyr-α), 114.1 (Tyr-ϵ), 130.0 (Tyr-δ), 158.6 (Tyr-ζ), 169.6 (Tyr-CO), 172.8 (CO_2Me), 177.6 (Ala-3-CO).

Dipeptide **5**[24] gave a very complicated ^{1}H NMR spectrum at 20 °C due to the presence of roughly equal amounts of four "stereoisomers" differing in rotation about the amide bonds. Heating a solution in nitrobenzene up to 120 °C caused the spectrum to simplify to the pattern expected with rapid rotation about the amide bonds; no significant change occurred on further heating to 180 °C. Barriers of 19.1 kcal/mol for the Tyr-2 amide bond and 16.3 kcal/mol for the Tyr-1 amide (actually urethane) bond[25] were most conveniently calculated from the coalescence temperatures observed for the Tyr-1 *N*-Me absorption as it went from

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(15) This very close agreement is fortuitous. Closer models are the peptides **4** and **5**, on which we have done variable temperature studies with the finding of a 17.5 kcal/mol barrier in the former and 19.1 kcal/mol (amide) and 16.3 kcal/mol (urethane) in the latter (See Experimental Section).

(16) The alanines, with N-H groupings instead of *N*-methyl, almost certainly have the usual *trans*-amide bonds throughout.

(17) For the Karplus curve of H–N–C_α–H in *trans*-peptides, see: Bystrov, V. F. *Prog. Nucl. Magn. Reson. Spectrosc.* **1976**, *10*, 46.

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(20) Some unnatural bouvardin derivatives which also differ only in the substituents on Tyr-5 and Tyr-6 have similar PS activities: methyldeoxybouvardin, 143–190% T/C at 0.25–2.0 mg/kg; bouvardin diacetate and deoxybouvardin acetate, 136–167% T/C at 0.25–2.0 mg/kg.

(21) Prof. J. P. Rosazza, University of Iowa, has prepared this compound by the microbial degradation of bouvardin (**2**). We thank him for informing us of his work and permitting us to refer to it prior to publication.

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four peaks to two at 38 °C ($\Delta\nu$ = 10 Hz) and two peaks to one at 94 °C ($\Delta\nu$ = 14 Hz).[14]

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^{205}Tl as an NMR Probe for the Investigation of Transferrin

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Abstract: Dithallium(III) transferrin and the monothallium derivative with bicarbonate as synergistic ion have been prepared and characterized. ^{205}Tl NMR spectroscopy is a reasonably good probe to monitor the occupancy of the two available binding sites. The signals for the two sites are well separated and have been assigned through titration with iron(III) at different pH values. Thallium shows larger affinity for the C-terminal site than for the N-terminal site all over the pH range investigated.

Human serum transferrin (TRN hereafter) is a glycoprotein of molecular weight 81 000[1] with two binding sites for iron(III).[1-5] The two sites are very apart in the rod-shaped protein and identified as C-terminal and N-terminal sites according to their location along the single polypeptide chain.[6,7] In vivo the two sites are occupied only at 30%.[4] The donor groups appear to be two tyrosinate anions and a number of histidines. Evidences of tyrosinate ligands stand on charge transfer spectra[8] and recently on a very appropriate model complex.[9] The presence of histidines is guessed from the observation of superhyperfine splitting in the EPR spectra of the copper(II) derivative[10] and from pulsed EPR techniques.[11] The bicarbonate ion is needed to form a stable ternary complex, and therefore it may be reasonably considered a donor group, as suggested also from the ^{13}C NMR data on the cobalt(III) derivative.[12] The overall coordination number presumably is at least six. Water proton NMR T_1^{-1} measurements, though they do not show definite evidence of exchangeable protons attached to the donor groups, are consistent with either H_2O or OH^- present in the coordination sphere.[9,13]

It is not clear whether the two sites are distinguishable from the physiological point of view;[1-5,14] however, the EPR spectra of the iron(III), copper(II), and oxovanadium(IV) derivatives have shown that the two sites are spectroscopically slightly nonequivalent,[10,15] whereas the distribution of a single metal ion over the two sites is dramatically pH dependent (for example at low pH only the C-terminal site is populated).[7] Substitution of iron(III) by a variety of metal ions has shown that also the size of the metal ion can distinguish between two sites; i.e., large ions such as neodymium(III) or praseodymium(III) can only enter the C-terminal site.[16]

Since the protein stabilizes the oxidation number three, we sought to introduce thallium(III) into the protein, not only to describe a new metal derivative but to provide a further spectroscopic tool for the investigation of the two sites. In principle, the ^{205}Tl NMR signal can be sensitive to the structural difference between the two sites, thus providing information on the metal distribution even in presence of other metal ions.

Experimental Section

Purified iron-free human serum transferrin (apo-TRN) was purchased from Sigma and was used without further purification. The lyophilized powder was dissolved in freshly bidistilled water, dialyzed twice against a 0.1 M $NaClO_4$ solution to remove possible traces of chelating agents,[17] and exhaustively dialyzed against bidistilled water. For spectroscopic studies apo-TRN was diluted up to a concentration of about 10^{-5} M.

The NMR samples were prepared by concentrating the apoprotein through ultradialysis up to $(1-2) \times 10^{-3}$ M. The concentration of apo-TRN was estimated spectroscopically, using $\epsilon_{278} = 9.23 \times 10^4$ M^{-1} cm^{-1}. All the experiments were performed in unbuffered solutions; the pH was adjusted to the required values by either addition of $NaHCO_3$ or by bubbling gaseous CO_2. The total concentration of bicarbonate and/or carbon dioxide was always in large excess with respect to the TRN sites concentration (0.03-0.3 M).

Thallium(III) solutions of approximate titer were prepared from $TlCl_3$ and adjusted to pH 3 by adding HCl. The actual thallium(III) concentration was determined by complexometric back-titrations. Excess EDTA was added to a known volume of $TlCl_3$ solution and the pH adjusted to 10 with ammonia buffer. Excess EDTA was then titrated by $MgCl_2$, using Eriochrome black T as indicator.

The electronic spectra were recorded on a Cary 17D spectrophotometer through the difference UV spectroscopy technique. ^{205}Tl NMR measurements were performed with a CXP Bruker spectrometer equipped with a 1.41-T electromagnet operating at 34.7 MHz; quadrature detection and a standard phase alternated pulse sequence (PAPS) were employed.

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