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Synthesis and Characterization of Dimeric Lumazines

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Summary

Reduction of 1,3-dimethyllumazine **(1)** by zinc dust in Ac_2O/AcOH leads to the formation of 6-7 connected bis-lumazinyl derivatives. Depending on the reaction conditions either 7-(5-acetyl-5,6,7,8-tetrahydro-1.3-dimethyllumazin-6-yl)-1, 3-dimethyllumazin **(3)** or isomeric 7-(5-acetyl-5,6,7,8-tetrahydro-1, 3-dimethyllumazin-6-yl)-5-acetyl-5,6,7,8-tetrahydro-1, 3-dimethyllumazines **(2)** are formed. Treatment of 3 with MeOH/HCl gave **4** which is oxidized by air to a very stable 7,8-dihydro derivative 5 showing unexpected spectra properties. Further oxidation by $KMnO_4$ afforded 6,7-bis-1. 3-dimethyllumazinyl **(6)**. The isomeric 6,6-**(14)** and 7,7-bis-1, 3-dimethyllumazinyls **(21)** were also synthesized from 6-chloro-**(11)** and 7-chloro-1, 3-dimethyllu-mazine **(18)**, respectively, in a nickel catalyzed dimerization reaction. The various structures were proven by spectral means, elemental analyses and an X-ray analysis of **2**. Comparisons of the structural features are mainly based on UV data.

Introduction

Little is known about dimeric pteridines which have been suggested as potential structures on reductive coupling reactions (1,2). Mager and Berends (3) gave a first proof on the basis of mass spectral investigations that the reduction of 1,3-dimethyllumazine **(1)** by zinc dust in a mixture of glacial acetic acid and acetic anhydride leads to two dimeric acetylated lumazine derivatives. A direct linkage of the two nuclei through the 6 or 7 position was proposed but so far the true structures of this new type of bis-lumazinyls have not been elucidated. Since we have described the formation of 5,8-diacetyl-1,3-dimethyl-5,8-hydrolumazine (4,5) on treatment of **1** either with zinc or by cathodic electrochemical reduction in acetic anhydride alone anticipating a radical mechamism we got interested in these dimers formed allegedly by slightly modified reaction conditions. Furthermore we intended to elucidate their chemical structures by physical means and chemical transformations. These findings bear also some relationship to the reductions of pyrazine forming 1,4-diacyl -1,4-dihydropyrazines analogously (6,7).

Part CI : R. Eisele, K. Aritomo and W. Pfleiderer, Pteridines 1993, **4**, 178.

Results and Discussion

Repetition of Mager's experiment indicated by TLC that a complex mixture of compounds is formed during the applied reduction process. Tedious chromatographical separations allowed the isolation of the higher melting dimer whereas the data of the second component turned out to be different. In order to get more information about the various steps of the reductive coupling reaction less zinc dust was applied and also the reaction time was reduced to 1 hour. It was furthermore noticed that the reaction solution turned first to a yellow color which disappeared only under prolonged treatment by zinc. Work-up led to a yellow compound **(3)** in good yield and in form of nice crystals. We isolated obviously an intermediate since its further reduction with zinc in acetic acid/

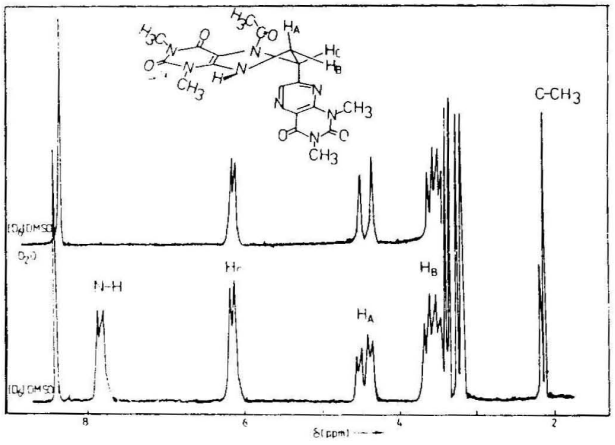


Figure 1. ^{1}H-NMR spectrum of **3** in D_6-DMSO and after H/D exchange.

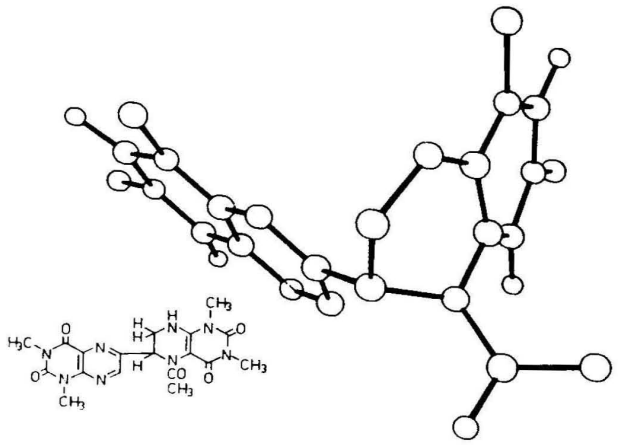
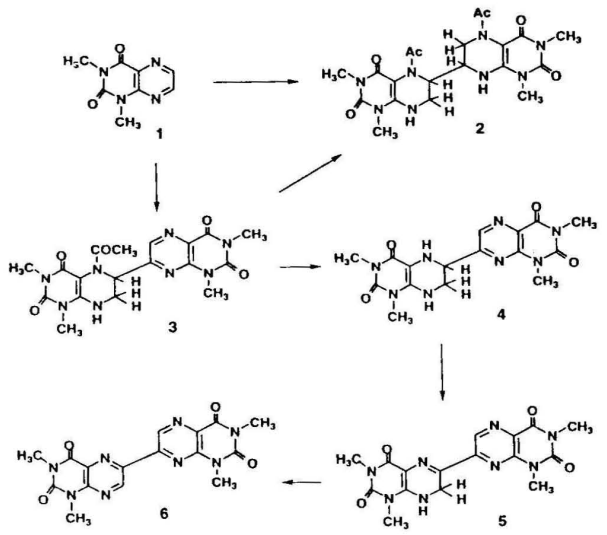


Figure 2. X-ray structure of **3**.

acetic anhydride gave two colorless isomers identical with the products formed as main components in the previous experiment.

Structure of **3** could be elucidated by physical, means. The elemental analysis led to the composition $C_{18}H_{20}N_8O_5xH_2O$ in combination with a mass spectrum indicating a mol mass m/e of 428 (M^+) and a characteristic fragment of 386 (M^+ -42) which is in agreemjent with the loss of ketene. The ^{1}H-NMR spectrum (Figure 1) showed convincingly the presence of one C-CH_3 and 4 different N-CH_3 signals as expected for a dimer carrying one acetyl group. The other proton signals, however, have been puzzling to some extent since besides a singlet at 8.40 ppm and one exchangable doublet (N-H) at 7.75 ppm only one other doublet at 6.12 ppm and two double doublets at 4.41 and 3.63 ppm could be detected. This simplified spectrum is only in agrement with the proposed structure of 5-acetyl-5,6,7,8-tetrahydro-1,3-dimethyl-6-(1,3-dimethyllumazin-7-yl)lumazine **(3)** if a special conformation eliminating by a perpendicular orien-



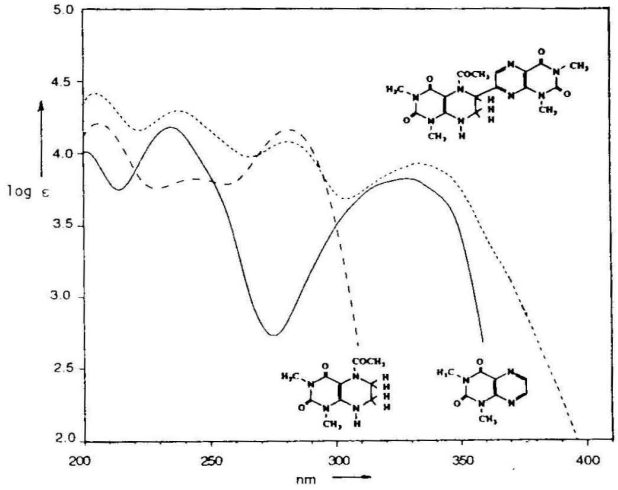
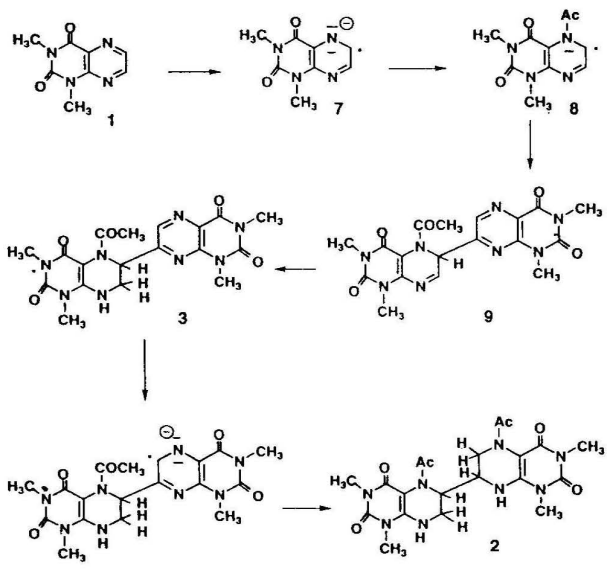


Figure 3. UV-spectra of **1**(———), **3**(– – –) and 5-acetyl-5,6,7,8-tetrahydro-1,3-dimethyllumazine (·····) in MeOH.

tation the coupling of one of the diastereotopic H-C (7A) with the adjacent H-C (6) and the N-H, respectively. H/D exchange gives further proof of this relations. Finally the anticipated structure could then been confirmed by an X-ray analysis of **3** showing exactly the proposed structural features (Figure 2).

It is furthermore noteworthy that the UV spectrum of **3** reflects nicely its composition since the two components 5-acetyl-5,6,7,8-tetrahydro-1,3-dimethyl-lmazine and 1,3-dimethyllumazine **(1)** superimpose with the dimeric structure very well as seen from the spectral comparison (Figure 3).

The formation of **3** can be explained by a radical mechanism giving rise to the regioselective 6-7 connection of the lumazine nuclei. In the first reduction step a radical anion **(7)** is formed whijch is trapped



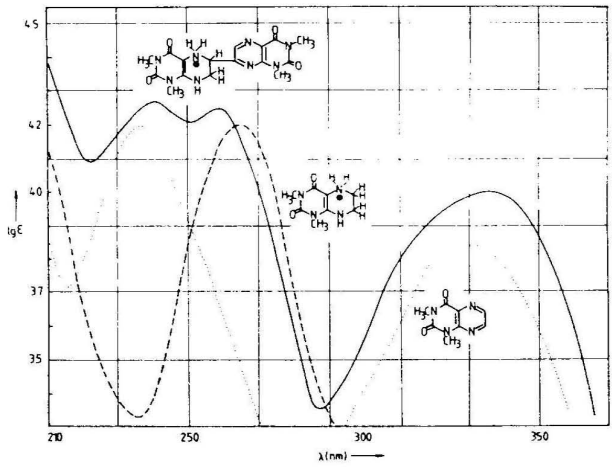


Figure 4. UV-spectra of **4**, **1** and 5,6,7,8-tetrahydro-1,3-dimethyllumazine in MeOH.

by acetylation at N-5 to give the neutral radical **8**. This radical then attacks another 1,3-dimethyllumazine molecule at position 7 in a nucleophilic radical substitution recation to **9** analogously to other radical acylations (8) which take place at the same site. Further reduction affords **3** since the partially reduced pyrazine ring will react more easily than its heteroaromatic counterpart.

Prolonged zinc treatment of **3** in AcOH/Ac_2O attacks also the second pyrazine ring in a similar manner which gave two 7-(5-acetyl-5,6,7,8-tetrahydro-1,3-dimethyl-lumazin-6-yl)-5-acetyl-5,6,7,8-tetrahydro-1,3-dimethyllumazines **(2)** identical with Mager's compounds and are most probable threo/erythro isomers regarding the central bond connection.

Further studies were concerned with the interconversion of **3** into 6,7-di-(1,3-dimethyllumazinyl) (6).

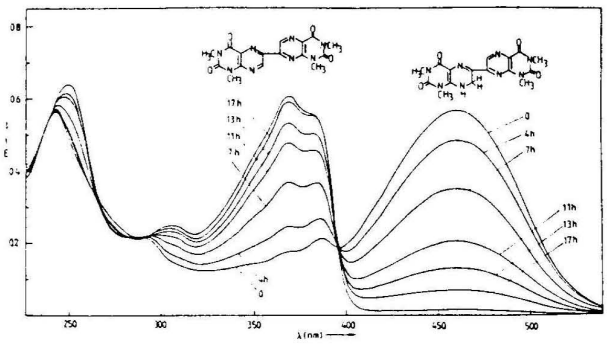


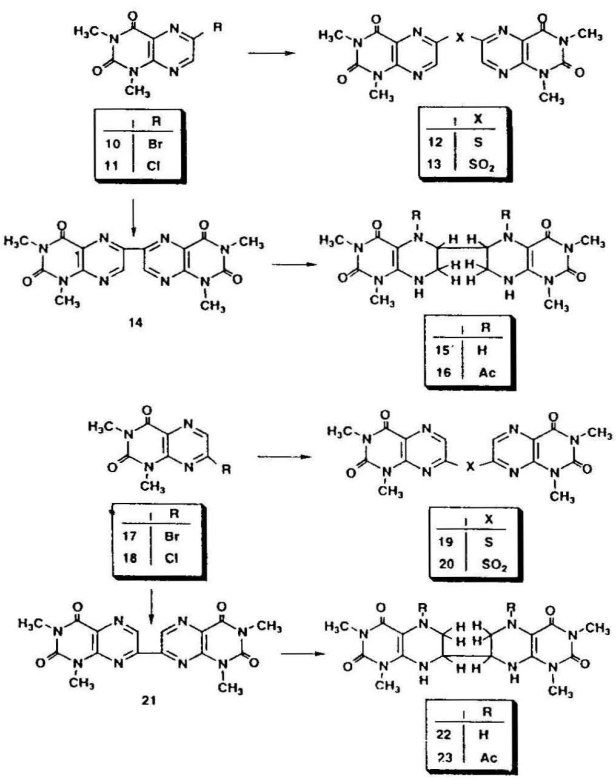
Figure 5. Autoxidation of **5** to **6** by oxygen in MeOH.

Treatment of **3** with methanolic HCl afforded the monohydrochloride salt of 7-(5,6,7,8-tetrahydro-1,3-dimethyllumazin-6-yl)-1,3-dimethyllumazine **(4)** the UV-spectrum of which can also been regarded as a composite of 1,3-dimethyllumazine (**1**) the 5,6,7,8-tetrahydro -1,3-dimethyllumazine cation (Figure 4).

Autoxidation of **4** resulted in the formation of a red-colored, less soluble compound showing a long wavelength absorption at 460 nm. This spectral feature is in accordance with the 7-(7,8-dihydro-1,3-dimethyllumazin-6-yl)-1,3-dimethyl-lumazine **(5)** structure indicating the presence of a merocyanine chromophore extending from the H-N (8) to the C=O (4) function of the heteroaromatic lumazine moiety. Further oxidation of compound **5** by permanganate or more slowly by air (Figure 5) led to the basic molecule of this series, 7-(1,3-dimethyllumazin-6-yl)-1,3-dimethyllumazine **(6)**.

The ^{1}H-MNR spectrum of this 6,7-dilumazine derivative indicates clearly the unsymmetrical structure of **6** in revealing 4 N-CH_3 and 2 H-C signals at different chemical shifts confirming additionally the proposed mechanism of formation. The assignments of the true stereochemistry to the isomeric 6,7-di-(5-acetyl-5,6,7,8-tetrahydro-1,3-dimethyllumazinyls **(2)** was not yet possible due to the complex signal patterns of the NMR spectra. Elemental analyses and mass spectra fit with the proposed structures and the materials do not contain crystal water as observed by Mager and Berends (3).

The successful synthesis of 6,7-di-(1,3-dimethyllumazinyl) **(6)** forced us to synthesize also the two other symmetrical 6,6- and 7,7-isomers, respectively. The first attempt consisted of a Raney-nickel desulfurization of di-(1,3-dimethyllumazin-6- **(12)** and -7-yl) sulfide **(19)** obtained from 6- **(10)** and 7-bromo-1, 3-dimethyllumazine **(17)** with thiourea. Loss of sulfur was observed under these condition but unfortunately only 1,3-dimethyllumazine (**1**) and no coupling to a dimer was observed. Also SO_2-extrusion reactions with the oxidation products **13** and **20** from **12** and



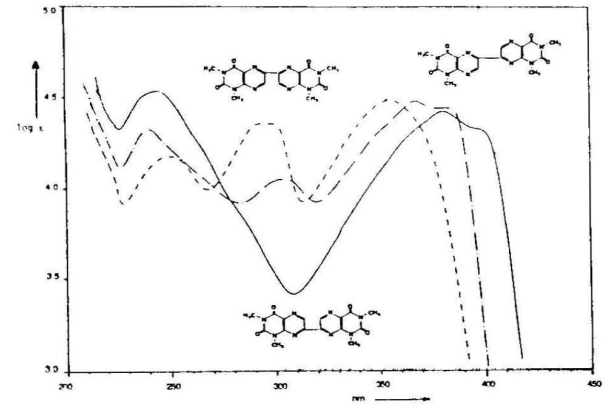


Figure 6. UV-spectra of **6** (–·–·–·), **14** (– – –) and **21** (————) in CH_2Cl_2.

19 have not been successful. We then tried various direct coupling reactions with 6- **(11)** and 7-chloro-1,3-dimethyllumazine **(18)**, respectively, starting with the Ullmann reaction and applying a broad variety of reaction conditions, but without success. Also the use of organometal complexes according to Caubere (8-11) which allows to modify the CRAs (complex reducing agents) to NiCRAL, for example, did not afford any progress. Finally, the $Ni(PPh_3)_4$ complex, prepared from nickel chloride and triphenylphosphane in DMF by treatment with zinc powder at 50℃ analogous to Tiecco et al. (12-14), initiated the anticipate coupling reaction in the expected manner. The yield of the dilumazinyl dimer formation is dependent on the reaction conditions since **11** gave at 50℃ in 2.5 hours 63% of **14** and **18** reacted similarly at 70℃ within 1.5 hours also to a 63% isolated yield of **21**. Reaction of a 1:1 mixture of **11** and **18** led as expected to a mixture of three compounds which were separated by tedious chromatographical means on preparative silica gel plates into three components identical with the dimers **6**,**14** and **21**. The ^{1}H-NMR spectra of **14** and **21** are very simple and show only one H-C and two N-CH_3 signals as expected from the symmetry in the structures. The UV spectra exhibit similarities in the long wavelength region with red-shifts from **14** to **6** to **21**, respectively (Figure 6).

Catalytic reduction of **14** and **21** afforded the corresponding octahydro derivatives **15** and **22** and subsequent acetylation by acetic anhydride led to stereoisomeric mixtures of di-(5-acetyl-5,6,7,8-tetrahydro-1,3-dimethyllumazin-6- **(16)** and -7-yl) **(23)**, respectively. The UV spectra fit very well with the monomeric 5-acetyl-5,6,7,8-tetrahydro-1,3-methyllumazine (15) but the NMR spectra are of complex nature to be analysed completely.

Experimental Part

General : -TLC : precoated silica gel thin-layer sheets F 1500 LS 254 and cellulose thin-layer sheets F 1440 LS 254 from Schleicher & Schüll. Prep. plates 20×20×0.2 cm silica gel PF_{254} from Merck. M.p. : Büchi apparatus, model Dr. Tottuli; no corrections. UV/VIS : Lambda 5 (Perkin Elmer); λ max (log ε) in nm; [ ] shoulder.. ^{1}H-NMR : Bruker WN-250; in δ (ppm) relative to TMS. MS : Finnigan MAT 312.

1,3-Dimethyllumazine (1) (16)

Heating of 0.1 g of either **12** or **19** in 50 ml of DMF with 4 g of wet Raneynickel for 1 hour afforded desulfurization. The catalyst was filtered off, DMF removed in vacuum and the residue recrystallized from little water to give 0.07 g (75%) of colorless crystals of m.p. 200°. The product is chromatographically identical with authentic 1,3-dimethyllumazine.

7-(5-Acetyl-5,6,7,8-tetrahydro-1,3-dimethyllumazin-6-yl)-5-acetyl-5,6,7,8-tetrahydro-1,3-dimethyllumazine (2)

a) In a mixture of 50 ml of AcOH and 50 ml of Ac_2O were dissolved 5 g of 1,3-dimethyllumazine (**1**) and then under boiling 7 g of zinc dust added gradually within 2 hours. After heating for another hour the insoluble precipitate was filtered off, the filtrate

evaporated and the residue coevaporated twice with EtOH. The solid was treated with 25 ml of hot acetone and filtered from little insoluble material. The filtrate was stored for 2 days whereby a precipitate (1.1 g) separated slowly. Two recrystallizations from EtOH gave 0.77 g (13%) colorless crystals of m.p.> 320°. Rf ($CHCl_3$/MeOH 4/1) 0.15. UV (MeOH) : [243, (4.04)], 282 (4.31). MS : 474 (M^+), 432 (M^+-CH_2CO). Anal. calc. for $C_{20}H_{26}N_8O_6$ (474.5) : C50.80, H 5.55, N23.32; found : C50.46, H5.65, N23.21.

Evaporation of the acetone filtrate and crystallization of the residue from 15 ml of MeOH gave 0.45 g which was further recrystallized from 50 ml of EtOH to give 0.26 g (4%) of the second component as colorless crystals of m.p.>320°. Rf ($CHCl_3$/MeOH 4:1) 0.40. UV (MeOH) : [243 (4.05)], 282 (4.29). MS : 474 (M^+), 432 (M^+-CH_2CO). Anal. calc. for $C_{20}H_{26}N_8O_6$ (474.5) : C50.80, H5.55, N23.32; found : C50.39, H5.60, N22.95.

b) In a mixture of 10 ml of AcOH and 10 ml of Ac_2O 0.6 g of 7-(5-acetyl-5,6,7,8-tetrahydro-1,3-dimethyllumazin-6-yl)-1,3-dimethyllumazine **(3)** were heated under reflux and then gradually 0.5 g of zinc dust added within 15 min. After boiling for 1 hour and cooling to room temperature was filtered from the zinc salts and the filtrate evaporated and then coevaporated with EtOH to dryness. The residue was treated with little MeOH to give 0.30 g of a substance mixture according to TLC. Purification was performed by chromatographical separation on preparative silica gel plates in $CHCl_3$/MeOH 9/1. The two main bands have been eluted and gave on evaporation the two isomers **2**, which have been chromatographically and spectrophotometrically identical with the two compounds described under a).

c) A solution of 0.1 g of **3** was dissolved in 20 ml of H_2O, 50l mg PtO_2 added and reduced under H_2 atmosphere in a shaking apparatus till two equivalents of H_2 were consumed. The catalyst was filtered off under Ar atmosphere, the filtrate evaporated to dryness and the residue treated with 5 ml of Ac_2O. After stirring for 2 hours was evaporated again and the residue, consisting of two isomers **2** again, separated by preparative silica gel chromatography as described under b).

7-(5-Acetyl-5,6,7,8-tetrahydro-1,3-dimethyllumazin-6-yl)-1,3-dimethyllumazine (3)

In a mixture of 35 ml of AcOH and 35 ml of Ac_2O were heated 3,5 g (0.0175 mol) of 1,3-dimethyllumazine **(1)** under reflux and then 1 g of zinc dust added gradually in small portions within 1 hours. After heating for 3 hours the precipitate was filtered hot, washed twice with hot AcOH and then the united filtrates evaporated in vacuum. The residue was treated with 80 ml of acetone to give 1.33 g of a crude yellow material. Recrystallization from 300 ml of H_2O gave 0.92 g (24%) of yellowish crystals of m.p.>310° (decomp.). UV (MeOH) : 239 (4.28), 283 (4.08), 334 (3.93). MS : 428 (M^+), 386 (M^+-CH_2CO). ^{1}H-NMR (D_6-DMSO) : 8.40 (s, 1H, H-C(6)); 7.75 (d, J=4 Hz, 1H, H-N); 6.12 (d, J=5 Hz, 1H, H-C(6'); 4.41 (dd, J=12 Hz, J=5 Hz, 1H, H-C(7'); 3.63 (dd, J=12 Hz, J=4 Hz, 1H, H-C(7"); 3.61 (s, 3H, N-CH_3); 3.29 (s, 3H, N-CH_3); 3.17 (s, 3H, N-CH_3; 3.11 (s, 3H, N-CH_3); 2,13 (s, 3H, $COCH_3$), Anal. calc. for $C_{18}H_{20}N_8O_5xH_2O$ (446.3) : C48.44, H4.97, N25.11; found : C 48.23, H4.89, N25.29.

7-(5,6,7,8-Tetrahydro-1,3-dimethyllumazin-6-yl)-1,3-dimethyllumazine hydrochloride (4)

In 20 ml of a saturated solution of methanolic HCl 0.5 g (1.12 mmol) of **3** were refluxed for 1 hour. It was evapotated to dryness and the residue treated with MeOH/ether to give 0.47 g (95%) of a colorless crystal powder of m.p.>330°. UV pH 1 : 241 (4.27), 259 (4.23), 337 (4.00).; pH 6.0 : 236 (4.32). 310 (4.15), [330 (4.09)]; pK : 2.37; MS : 386 (M^+). ^{1}H-NMR (D_6-DMSO) : Anal. calc. for $C_{16}H_{18}N_8O_4$. HCl. H_2O (440.8) : C43.59, H4.80, N25.42; found : C43.30, H 4.68, N25.61.

7-(7,8-Dihydro-1, 3-dimethyllumazin-6-yl)-1,3-dimethyllumazine (5)

A solution of 0.44 g (1 mmol) of **4** in 40 ml of H_2O was stirred at room temperature for 3 days while a slow stream of oxygen was bubbled throught the reaction solution. An orange colored precipitate separated and was collected. The less soluble material was treated with hot MeOH, filtered and dried in the oven at 100° to give 0.276 g (72%) of an orange powder of m.p.>330°. UV (MeOH) : 249 (4.40), [285 (3.92)], 335 (3.58); 460 (4.39). pH 7.0 : 249 (4.38), [285 (3.95)], [370 (3.84)], 390 (3.92), 459 (4.31); pH 13 : 227 (4.29), 282 (4.18), 350 (3.87), [384 (3.72)], 545 (4.30); pK : 10.51; ^{1}H-NMR (D_6-DMSO) : 9.14 (s, 1H, H-C(6)); 7.94 (bs, 1H, H-N); 4.59 (s, 2H, H-C(7)); 3.58 (s, 3H, N-CH_3); 3.36 (s, 3H, N-CH_3); 3.33 (s, 3H, N-CH_3); 3.22 (s, 3H, N-CH_3). Anal. calc. for $C_{16}H_{16}N_8O_4$ (384.4) : C49.99, H4.20, N 29.16; found : C49.96, H3.91, N29.49.

7-(1,3-Dimethyllumazin-6-yl)-1,3-dimethyllumazine(6)

a) A solution of 0.22 g (0.5 mmol) of **4** in 23 ml

of DMF was heated to 60° and under stirring 2 ml of 30% H_2O_2 gradually added. After stirring over night 20 ml of H_2O was added and the precipitate collected. Recrystallization from DMF with little charcoal gave 0.145 g (76%) of yellowish crystals of m.p.>330°.

b) A solution of 0.22 g (0.5 mmol) of **4** in 50 ml of H_2O was oxidized under stirring by dropwise addition of 0.15 g $KMnO_4$ in 20 ml of H_2O. The mixture was heated to 50° for 2 hours and then the precipitate (MnO_2+substance) collected. After drying the solid was continously extracted by $CHCl_3$ in a Soxhlet apparatus for 2 days. The $CHCl_3$ extract was evaporated to dryness and the residue recrystallized from DMF under addition of little H_2O to give 0.14 g (73%) of yellowish crystals of m.p.>330°. UV (CH_2Cl_2) : 242 (4.34), 304 (4.05), 371 (4.47), [390 (4.44)]. MeOH : 211 (4.32), 241 (4.34), 304 (4.03), 369 (4.47), [381 (4.44)]. MS : 382 (M^+). ^{1}H-NMR (D_6-DMSO) : 9.70 (s,1H, H-C(7)); 9.36 (s, 1H, H-C(6)); 3.72 (s, 3H, N-CH_3); 3.66 (s, 3H, N-CH_3); 3.42 (s, 3H, N-CH_3); 3.40 (s, 3H, N-CH_3). Anal. calc. for a $C_{16}H_{14}N_8O_4$ (382.3) : C50.26, H3.69, N29.31; found : C50.15, H3.69, N29.20.

Bis (1,3-dimethyllumazin-6-yl)-sulfide (12)

A mixture of 0.82 g (3 nmol) of 6-bromo-1.3-dimethyllumazine **(10)** (17) and 0.5 g thiourea were refluxed in 40 ml of EtOH for 24 hours. The precipitate was collected and gave after drying at 100° 0.32 g (51%) of a yellowish crystal powder of m.p. 308~310°. UV (MeOH) : 245 (4.35), 269 (4.34), 360 (4.11). ^{1}H-NMR (D_6-DMSO) : 9.00 (s, 2H, H-C (7)); 3.92 (s, 3H, N-CH_3); 3.68 (s, 3H, N-CH_3). Anal. calc. for $C_{16}H_{14}N_8O_4S$ (414.3) : C46.38, H3.40, N27.03; found : C 46.40, H3.43, N27.06.

Bis-(1,3-dimethyllumazin-6-yl)-sulfone (13)

A solution of 0.2 g of **12** in trifluoroacetic acid and a solution of 20 mg of $Na_2WO_4 \times 2H_2O$ were united and to the resulting suspension 4 ml of 30% H_2O_2 added dropwise. After stirring for 2 hours the clear solution was evaporated and then the residue treated with 20 ml of H_2O to form a crystalline solid. Washing with EtOH and drying yielded 0.185 g (85%) of yellowish crystals of m.p.>340°. UV (CH_3CN) : 253 (4.47), [275 (4.33)], 336 (4.26). MS : 446 (M^+). ^{1}H-NMR (D-TFA) : 9.74 (s, 2H, H-C(7,7')); 3.95 (s, 6H, N-CH_3); 3.66 (s, 6H, N-CH_3). Anal. calc. for $C_{16}H_{14}N_8O_6SxH_2O$ (446.3) : C41.38, H3.74, N24.13; found : C41.41, H3.43, N24.21.

Bis-(1,3-dimethyllumazin-6-yl) (14)

To a solution of 0.48 g of $NiCl_2 \times 6H_2O$ in 30 ml of DMF 2.08 g of triphenyl-phosphane was added whereby the dark green color changed to dark blue. The solution was heated to 50° and under nitrogen atmosphere 0.13 g zinc powder added gradually. After 1 hour of stirring 0.45 g (2 mmol) of 6-chloro-1,3-dimethyl-lumazine **(11)** (17) were added and after treatment for another 2.5 hours at 50° the warm reaction solution poured into 100 ml of 1N ammonia. The resulting suspension was shaken twice with 200 ml of ether and then extracted continuously in a perforator with $CHCl_3$ over night. The $CHCl_3$-phase was dried over Na_2SO_4, evaporated and the residue recrystallized from 140 ml of DMF+7 ml of H_2O to give 0.24 g (63%) of a yellowish crystal powder of m.p.>350°. UV (CH_2Cl_2) : 250 (4.18), 300 (4.38), 356 (4.50). MS : 382 (M^+). ^{1}H-NMR (D-TFA) : 10.07 (s, 2H, H-C (7,7')); 4.00 (s, 6H, N-CH_3); 3.79 (s, 6H, N-CH_3). Anal. calc. for $C_{16}H_{14}N_8O_4$ (382.3) : C50.26, H3.69, N 29.31; found : C50.28, H3.75, N29.32.

Bis-(5,6,7,8-tetrahydro-1,3-dimejthyllumazin-6-yl) Dihydrochloride (15)

In 20 ml of TFA 40 mg of PtO_2 were reduced in a shaking apparatus under hydrogen atmosphere. Then a solution of 0.191 g (0.5 mmol) of 14 in 30 ml of TFA added and the reduction continued for 8 hours. It was filtered from the catalyst and the filtrate added slowly with stirring into methanolic HCl. The mixture was evaporated to dryness and the residue treated with ether. The precipitate was collected, treated with MeOH again and then ether added dropwise to give after drying 0.118 (51%) of a colorless powder of m.p.>310° (decomp.). UV (pH 0.0) : 205 (4.40), 265 (4.39); (pH7) : 237 (4.08), 299 (4.25). MS : 390 (M^+). Anal. calc. for $C_{16}H_{22}N_8O_4 \times 2HCl \times 2H_2O$ (499.3) : C38.38, H5.65, N22.44; found : C38.16, H5.42, N22.48.

Bis-(5-acetyl-5,6,7,8-tetrahhdro-1,3-dimethyllumazin-6-yl) (16)

In TFA were reduced 0.19 g (0.5 mmol) of **14** analogously to the preceeding procedure. After evaporation to dryness 20 ml of Ac_2O was added and the mixture heated under gentle reflux for 30 min. Again evaporation to dryness and recrystallization of the residue from H_2O gave 0.147 g (62%) of colorless crystals of m.p.>330°. UV (MeOH) : 207 (4.20), [250 (3.79)], 281 (4.21). MS : 474 (M^+). Anal.calc. for a $C_{20}H_{26}N_8O_6 \times 2H_2O$ (510.5) : C47.05, H5.95, N21.95; found : C47.19, H5.76, N22.11.

Bis-(1,3-dimethyllumazin-7-yl)-sulfide (19)

a) A mixture of 0.82 g (3 mmol) of 7-bromo-1, 3-dimethyllumazine (**17**) (17) and 0.5 g thiourea in 40 ml of EtOH was refluxed for 5 hours. During the reaction 5 drops of triethylamine were added. After cooling the precipitate was collected, washed and dried at 100° to give 0.38 g (61%) of a yellowish powder of m.p.>275° (decomp.).

b) A mixture of 0.224 g (1 mmol) of 7-mercapto-1, 3-dimethyllumazine (18) and 0.276 g (1 mmol) of 7-bromo-1,3-dimethyllumazine (**17**) in 30 ml of EtOH was heated under reflux for 4 hours. During the reaction 5 drops of triethylamine were added gradually. The precipitate as collected, washed with EtOH and dried to give 0.397 g (96%) of a chromatographically pure yellowish powder of m.p.>275° (decomp.).

c) A mixture of 0.67 g (3 mmol) of 7-chloro-1,3-dimethyllumazine **(18)** (17) and 0.5 g of thiourea was refluxed for 8 hours under gradual addition of 5 drops of triethylamine. The resulting precipitate was collected, washed with EtOH and dried to give 0.58 g (93%) of a yellowish powder of m.p.>275° (decomp.). UV (CH_2Cl_2) : 228 (4.43), [346 (4.20)], 376 (4.42). ^{1}H-NMR (D_6-DMSO) : 8.98 (s, 2H, H-C(6)); 3.74 (s, 3H, N-CH_3); 3.66 (s, 3H, N-CH_3). Anal. calc. for $C_{16}H_{14}N_8O_4S$ (414.3) : C46.38, H3.40, N27.06; found : C 46.55, H3.42, N27.06.

Bis-(1,3-dimethyllumazin-7-yl)-sulfone (**20**)

Solutions of 0.207 g (0.5 mmol) of **19** in 20 ml of TFA and of 20 mg of $Na_2WO_4 \times 2H_2O$ in 10 ml of H_2O were united and the resulting suspension treated with 4 ml of 30% H_2O_2 by dropwise addition. After stirring for 1 day at room temperature was evaporated and the residue stirred with 20 ml of H_2O. The solid was collected and gave after recrystallization from 20 ml of DMF/H_2O 1/1 0.152 g (70%) of yellow crystals of m.p.>260° (decomp.). UV (CH_3CN) : 245 (4.18), 354 (4,46). MS : 446 (M^+). ^{1}H-NMR (D-TFA) : 9.57 (s, 2H, H-C (6,6')), 3.78 (s, 6H, N-CH_3), 3.69 (s, 6H, N-CH_3). Anal. calc. for $C_{16}H_{14}N_8O_6SxH_2O$ (464.3) : C42.25, H3.52, N24.65; found : C42.22, H3.57, N24.46.

Bis-(1,3-dimethyllumazin-7-yl) (21)

Analogous to procedure **14** 0.45 g (2 mmol) of 7-chloro-1,3-dimethyllumazine (**18**) (17) were heated to 70° for 1.5 hours. Work-up by extraction was the same and gave after recrystallization of the reaction product from 140 ml of DMF by addition of 7 ml of H_2O 0.24 g (63%) of yellowish crystals of m.p.>350°. UV (CH_2Cl_2) : 225 (4.35), 246 (4.54), 382 (4.42), [398 (4.32)]. MS : 382 (M^+). ^{1}H-NMR (D-TFA) : 9.90 (s, 2H, H-C (6,6')); 4.07 (s, 6H, N-CH_3); 3.76 (s, 6H, N-CH_3). Anal. calc. for $C_{18}H_{14}N_8O_4$ (382.2) : C50.29, H3.69, N29.29; found C49.96, H3.82, N29.20.

Bis-(5,6,7,8-tetrahydro-1,3-dimethyllumazin-7-yl) Dihydrochloride (22)

In 20 ml of TFA 30 mg of PtO_2 were reduced in a shaking apparatus under hydrogen atmosphere. Then a solution of 0.191 g (0.5 mmol) of 21 in 20 ml TFA was added and the reduction continued for 8 hours. After 4 hours the theoretical amount of H_2 was taken up. The catalyst was filtered off and the filtrate dropwise added to a solution of methanolic HCl. After evaporation the residue was treated with ether, then dissolved in MeOH and finally added to ether with stirring forming a precipitate. Drying in vacuum yielded 0.127 g (66%) of a colorless powder of m.p.> 310° (decomp.). UV (pH 1.0) : 205 (4.43); 267 (4.43); (pH 7.0) : [247 (3.86)], 300 (4.26). MS : 390 (M^+). ^{1}H-NMR (D-TFA) : 5.12 (bs, 2H, H-C (7,7')); 4.36+4.12 (dd, 4H, H-C (6,6')); 3.77 (s, 6H, N-CH_3); 3.60 (s, 6H, N-CH_3)). Anal. calc. for $C_{16}H_{22}N_8O_4 \times 2HCl \times 2H_2O$ (499.3) : C38.48, H5.65, N22.44; found C38.53, H5.38, N22.32.

Bis-(5-acetyl-5,6,7,8-tetrahydro-1,3-dimethyllumazin-7-yl) (23)

Analogous to the preceding procedure 0.191 g (0.5 mmol) of 21 were reduced in TFA. After filtration of the catalyst the filtrate was evaporated to dryness and the residue heated under gentle reflux in 20 ml of Ac_2O for 30 min. Evaporation and treatment of the remaining solid with acetone yielded 0.167 g (76%) of a colorless powder of m.p.>330°. UV (MeOH) : 211 (4.22), 246 (3.82), 287 (4.24). MS : 474 (M^+). ^{1}H-NMR (D-TFA) : 5.30 (d, 2H, H-C (6)); 3.83 (s, 6H, N-CH_3); 3.57 (s, 6H, N-CH_3); 3.66 (s, 2H, H-C (7,7')); 3.06 (d, 2H, H-C (6')); 2.43 (s, 6H, Ac). Anal. calc. for $C_{20}H_{26}N_8O_6 \times 2H_2O$ (510.4) : C47.05, H5.92, N21.95; found : C46.88, H5.78, N21.80.

Coupling of 6-chloro-(11) and 7-chloro-1,3-dimethyllumazine (18) to 6, 14 and 21

Analogous to procedure **14** 0.226 g (1 mmol) of **11** and 0.226 g (1 mmol) of **18** were reacted at 50° for 2 hours. Work-up by extraction gave a mixture of three compounds which was separated by preparative silica gel chromatography on plates (20×20×0.2 cm) in toluene/EtOAc (4/1) to give 60 mg (16%) of **21** (Rf 0.44), 20 mg (5%) of **6** (Rf 0.54) and 0.115 g (30%) of **14** (Rf 0.70). All three compounds were chromato-

graphically and spectrophotometrically identical with the authentic materials.

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Synthesis of N-(4-propargylaminobenzoyl)-L-glutamates

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Summary

To facilitate the rather labour consuming synthesis of antifolate γ-oligo-L-glutamates including those with the propargyl group at position N^{10}, two synthons possessing this group, α-t-butyl γ-methyl *N*-(4-propargylaminobenzoyl)-L-glutamate (1) and α-t-butyl *N*-(4-propargylaminobenzoyl)-L-glutamate (2) have been elaborated (Scheme 2) and characterized by melting points, elemental analysis, NMR, TLC and HPLC. They are of purity >98.5% by the last method.

Key words: *N*-(4-propargylaminobenzoyl)-L-glutamates, N^{10}-propargyl antifolates, ^{1}H NMR spectra, HPLC, Short column chromatography.

Introduction

A potent thymidylate synthase (EC 2.1.1.45) inhibitor, *N*-[4-[(2-amino-3,4-dihydro-4-oxo-6-quinazolinyl)methyl]prop-2-ynyl-amino]benzoyl]-L-glutamic acid (Scheme 1; synonyms : N^{10}-propargyl-5,8-dideazafolic acid, CB 3717, PDDF, ICI 155, 387, NSC 327182) (1) continues to be the lead structure in designing and synthesising related compounds, which retain the benficial impact of the N^{10}-propargyl group on binding the enzyme, but at the same time have more desirable biochemical and pharmacological profiles than the parent CB 3717 (2-9). To determine the detailed biological and pharmacological properties of these antifolic analogues indispensable are appropriate investigations of their γ-conjugates with glutamic acid, g-oligo(glutamic acid)s or another amino acid(s) (6,10-13). They can be obtained (Scheme 1) according to either of the strategies of peptide chain building (14), convergent (11,13) or step by step (10,12). Both are labour consuming and for both, labour saving synthons of BC type are desirable. The convergent plan requires compounds BC with both glutamic acid carboxyl groups protected, each in different manner. In the stepwise strategy, the glutamic acid γ-carboxyl group should be free. Herein we report on the preparation of α-t-butyl γ-methyl N-(4-propargylaminobenzoyl)-L-glutamate (1) and α-t-butyl N-(4-propargylaminobenzoyl)-L-glutamate (2) (Scheme 2), some valuable intermediates in the synthesis of N^{10}-propargyl-5,8-dideazafolic acid analogues shown in Scheme 1 as well as of other analogues of N^{10}-propargylfolic acid.

Experimental Part

General. Purified solvents (Polskie Odczynniki Chemiczne) were stored over drying agents. Organic solutions were dried with anhydrous Na_2SO_4. Solvents from them and from fractions after column chromatography were removed in vacuo on a rotatory evaporator at a bath temperature not exceeding 30°C unless otherwise indicated. The reactions were monitored and the homogeneity of products was checked by TLC (DC Alufolien Kieselgel 60 No 5553 Merck) in systems: (A) chloroform-methanol-acetic acid (95 : 5 : 3) and (B) chloroform-methanol-conc. ammonia

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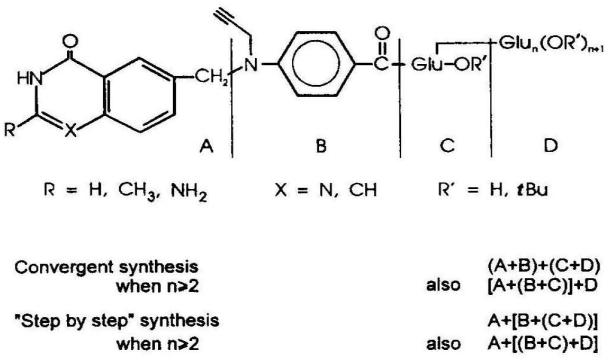
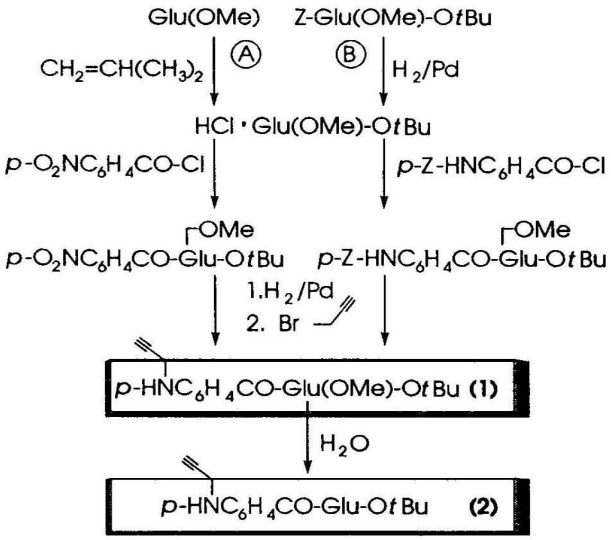
Abbreviation used : Glu, glutamic acid; *t*Bu, *t*-butyl; Me, methyl; Z, benzyloxycarbonyl.



Scheme 1. General structure of γ-oligo-L-glutamates of N^{10}-propargyl-5,8-dideazafolic acid (R=NH_2, R'=H, X=N and n=0) and its analogues. Division has been shown into moieties A, B, C and D, Which can be linked according to the convergent or stepwise peptide synthesis strategy.



Scheme 2. Synthetic scheme of α-t-butyl γ-methyl N-(p-propargylbenzoyl)-L-glutamate **(1)** and α-t-butyl N-(p-propargylbenzoyl)-L-glutamate **(2)**.

(6 : 5 : 1). Spots were visualized with chlorine-KI-tolidine reagent and ninhydrin. M.ps. were determined on a Boëtius heating block and are given uncorrected. HPLC analyses were performed on a Beckman "System Gold" for Methods Development consisting of a Model 126 programable module, a Model 168 diode array detector operating at 210 nm, a Model 210A injection valve with a 5 μl loop and a PC386SX (Wearnes) with "System Gold" version 5.1 software for data collection and controller function. Samples, dissolved in acetonitrile, a 150×4.6 mm Alltima C18 5 μm column, purchases from Alltech and a mobile phase with a flow rate 1 ml/min were used. Elemental analyses were performed on a Perkin-Elmer analyzer. ^{1}H NMR spectra were taken on a 100 MHz Tesla BS 567 spectrometer in CD_3COCD_3 with tetramethylsilan as internal standard.

α-t-butyl γ-methyl L-glutamate hydrochloride

Method (A). To stirred dioxane (250 ml) with conc. H_2SO_4 (25 ml), cooled to 10℃, liquid isobuten (250 ml) was poured, the total stirred at 5℃ for 20 min and Glu (OMe) (Bachem) (24.2 g, 150 nmol) introduced. Stirring was continued at 20℃ for 4 h, the obtained clear solution cooled to 0℃ was added. The resulted solution was extracted with diethyl ether (3×200 ml). The organic pool was washed with cooled water and cooled brine (200 ml each). After drying, ether (300 ml) was evaporated at a bath temperature not exceeding 10℃, 2N etheric HCl (50 ml) added dropwise with intense stirring and after 20 min the formed precipitate was filtered off, washed with cooled ether and hexane (2×100 ml each) and dried in vacuo for 24 h to give 22.8 g (60% yield) of HCl.Glu(OMe)-OtBu of m.p. 131～132℃. R_f(B) 0.85.

Method (B). Through a solution of Z-Glu (OMe)-OtBu (15) (5.27 g, 15 mmol) in methanol (45 ml) with 10% Pd/C (1.1 g), wetted with ethanol, hydrogen was bubbled for 20 min. The catalyst was filtered off, washed with methanol and the filtrate was concentrated at a bath temperature not exceeding 15℃ to about 15 ml and cooled to 0℃. 2N Etheric HCl (7 ml) was added dropwise followed by petroleum ether (20 ml) and after 30 min the resulted precipitate was filtered off, washed with cooled ether and hexane (2×10 ml each) and dried in vacuo for 24 h to furnish 3.55 g (93% yield) of HCl.Glu(OMe)-OtBu of m.p. 129～130℃. R_f(B) 0.85.

α-t-butyl-r-methyl N-(p-benzyloxycarbonylaminobenzoyl)-L-glutamate

To a stirred solution of p-Z-NHC_6H_4CO-Cl (16) (20.35 g, 70 mmol) and HCl.Glu(OMe)-OtBu (16.70 g, 70 mmol) in dioxane (70 ml), cooled to 5℃, tri(n-butyl)amine (33.60 ml, 140 mmol) was being added dropwise for 20 min and stirring was continued at 20 ℃ for 20 min. Ethyl acetate was added (750 ml), washed with water, 0.1N HCl, brine, 0.4M K_2CO_3 and brine (3×150 ml each), dried and evaporated. The residue, crystallized from CH_2Cl_2/petroleum ether gave the product (31.25 g, 95% yield) of m.p. 108～108.5℃. Rf(A) 0.50. HPLC, 0.1% trifluoroacetic acid : acetonitryl (30 : 70): tR=3.38 min; 98.9% purity. ^{1}H-NMR: 7.95 (d, 2H, Ar2'6'), 7.65 (d, 1H, Ar-CONH), 7.40 (s, 5H, Ar), 7.30 (bs, 1H, NH), 6.90 (d, 2H, Ar3'5'), 4.85 (s, 2H, $ArCH_2$), 4.20 (m, 1H, aCH), 3.60 (s, 3H, CH_3), 2.50-3.00 (m, 4H, CH_2-CH_2), 1.45 (s, 9H, $C(CH_3)_3$). Anal. calc. for $C_{25}H_{30}N_2O_7$ (470.2) : C

63.80, H 6.43, N 5.96; found: C 64.08, H 6.61, N 6.10.

α-t-butyl γ-methyl N-(p-nitrobenzoyl)-L-glutamate

To a stirred solution of p-$O_2NC_6H_4CO$-Cl (Aldrich) (1.69 g, 9 mmol) and HCl.Glu(OMe)-OtBu (2.28 g, 9 mmol) in dioxane (9 ml), cooled to 5℃, tri(n-butyl) amine (4.3 ml, 18 mmol) was being added dropwise for 20 min and stirring was continued at 20℃ for 20 min. The same work-up as above furnished a colourless oil (3.13 g, 95% yield). R_f(A) 0.37.

α-t-butyl γ-methyl N-(p-propargylbenzoyl)-L-glutamate (1)

Through a solution of p-Z-NHC_6H_4CO-Glu(OMe)-OtBu or p-$O_2NC_6H_4CO$-Glu(OMe)-OtBu (30 mmol each) in methanol (120 ml) with 10% Pd/C (20% weight of a substrate), wetted with ethanol, hydrogen was bubbled for 1.5 h. The catalyst was filtered off, washed with methanol, the filtrate evaporated to an oil, diethyl ether was added and evaporated to give, after drying in vacuo for 12 h, p-$H_2NC_6H_4CO$-Glu (OMe)-OtBu (29.7 mmol). This was dissolved in dimethylacetamide (30 ml) and $CaCO_3$ (45 mmol) and a 80% toluene solution of propargyl bromide (Aldrich) (3.6 ml, 32 mmol) were introduced. The mixture was stirred in the dark for 120 h. Ethyl acetate was added (500 ml), washed with water (4×300 ml), 0.1 M $NaHCO_3$ (2×300 ml) and brine (3×200 ml), dried and evaporated. The residue, dissolved in $CHCl_3$ (20 ml) was applied to a short column (17) (F 5 cm, silicagel Merck No 7736, 300 g) and eluted with chloroform of increasing amount of ethyl acetate from 1% to 6% (6×250 ml). The appropriate fractions were evaporated to give 1 (6.37 g, 56% yield). m.p. 131-133℃. R_f(A) 0.66. HPLC, 0.1% trifluoroacetic acid : acetonitryl (50 : 50): tR=5.85 min; 99.0% purity. ^{1}H-NMR: 7.90 (d, 2H, AR2'6'), 7.170 (d, 1H, ArCONH), 6.85 (d, 2H, Ar3'5'), 6.00 (t, 1H, NH), 4.60 (m, 1H, aCH), 4.10 (d, 2H, NCH2), 3.65 (s, 3H, CH3), 3.00 (t, 1H, >CH), 2.05-2.60 (m, 4H, CH_2-CH_2), 1.50 (s, 9H, $C(CH_3)_3$). Anal. calc. for $C_{20}H_{26}N_2O_5$ (374.2) : C 64.14, H 7.00, N7.48; found : C64.54, H7.36, N7.39.

a-t-butyl N-(p-propargylbenzoyl)-L-glutamate (2)

To a stirred solution of **1** (6.37 g, 17 mmol) in acetone (35 ml) at 30℃, 1N NaOH (20.5 ml) was added. Stirring was continued at 30℃ for 20 min, 1N HCl (20.5 ml) added and acetone evaporated. Water was added and extracted with ethyl acetate (2×100 ml). The organic pool was washed with water and brine (3×50 ml each), dried, evaporated and crystallized from H_2Cl_2/petroleum ether to furnish **2** (4.90 g, 80% yield) of m.p. 141～142℃ R_f(A) 0.37. HPLC, 0.1% trifluoroacetic acid : acetonitryl (50 : 50): tR= 3.05 min; 98.5% purity. ^{1}H-NMR: 7.85 (d, 2H, Ar2'6'), 7.60 (d, ^{1}H, ArCONH), 6.80 (d, 2H, Ar3'5'), 6.10 (bs, 1H, NH), 4.65 (m, 1H, aCH), 4.05 (d, 2H, NCH2), 2.95 (t, 1H, ≡CH), 2.00～2.75 (m, 4H, CH_2-CH_2), 1.50 (s, 9H, C $(CH_3)_3$). Anal. calc. for $C_{19}H_{24}N_2O_5$ (360.2) : C64.30, H 6.72, N7.78; found : C63.40, H7.15, N7.87.

Results and Discussion

α-t-Butyl γ-methyl N-(p-propargylbenzoyl)-L-glutamate **(1)** can be obtained in two ways, A or B (Scheme 2). The advantage of the former are commercial substrates. Drawbacks are (i) cool edulcorations in preparing Glu (OMe)-OtBu due to numerous side-reactions and (ii) the product of p-nitrobenzoylation, p-$O_2NC_6H_4CO$-Glu (OMe)-OtBu being an oil. Way B is very easy, fast and highyielding (83% of p-Z-HNC_6H_4CO-Glu(OMe)-OtBu overall), but fully-blocked substrates are used, the synthesis of which has however been well-elaborated (15, 16). We eventually recommend as the most practical Glu(OMe) and to acylate the diester with p-Z-HNC_6H_4COCl. The two steps should be best performed one by one, because as Glu(OMe)-OtBu, also its hydrochloride is not stable. After one week of standing the latter in a refrigerator, some decomposition has already been visible by TLC (system B). Hydrogenation of both p-nitrobenzoyl- and p-benzyloxycarbonyl-glutamate is very fast and poses no problem. Propargylation proceeds with by-formation of the dialkyl derivative and the post-reaction mixture requires to be chromatographed. L-Glutamate **1** is easily saponified to give α-t-butyl N-(p-propargylbenzoyl)-L-glutamate (**2**). Compounds **1** and **2** have been characterized by melting points, elemental analysis, NMR. TLC and HPLC. They are of purity >98.5% by the last method. The application of **2** for the convenient synthesis of a series of t-butyl N-(4-propargylaminobenzoyl)-g-oligo (L-glutamate)s will be published in a forthcoming paper.

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Serum Neopterin and Angiotensin-converting Enzyme in Cancer Patients

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Summary

An elevation of serum angiotensin-converting enzyme (ACE) has been reported in some disorders associated with immune activation, e.g. sarcoidosis, HIV infection or ulcerative colitis. Both endothelial cells and macrophages are thought to be the possible source of increased ACE levels. We have investigated serum ACE and neopterin, an indicator of macrophage activation, in 23 patients with solid tumors as well as in 8 controls. Similar ACE levels have been observed in the cancer patients and the controls (2.6 ± 2.1 vs 3.4 ± 1.1 μkat/1, while neopterin has been significantly higher in the cancer patients (15.4 ± 12.6 vs 7.2 ± 2.2 nmol/l, $P<0.02$). No correlation has been observed between serum ACE and neopterin. We conclude that an elevation of ACE is not present in cancer patients. Macrophage activation in cancer is not accompanied by increased ACE release.

Key words: Angiotensin-converting enzyme, Cancer, Neopterin.

Introduction

Angiotensin-converting enzyme (ACE) is produced mainly by endothelial cells and plays a key role in regulating the metabolism of vasoactive peptides, i.e. the conversion of angiotensin I to angiotensin II and inactivation of bradykinin (1). ACE production was also demonstrated in macrophages and an increase of serum ACE levels resulting from macrophage activation has been associated with sarcoidosis (2). More recently, an increase in serum ACE has been also reported in patients with HIV infection and ulcerative colitis (3, 4), but the cellular source of ACE in these disorders remains to be determined.

Macrophage activation in many clinical situations, including the mentioned disorders, has been associated with a rise in serum and urine neopterin (5, 6,7,8). Increased neopterin production in these conditions is known to result mainly from the action of interferon-gamma, a cytokine released by T-lymphocytes (9), on macrophages, and is now widely used to assess immune activation (5). Elevated urine and serum neopterin levels have been described in many different tumors (5,10,11,12), reflecting the macrophage activation induced by interferon-gamma.

The aim of the present study was to determine whether the rise in serum neopterin in cancer patients is accompanied by increased serum ACE levels.

Material and Methods

Patients

Twenty-three previously untreated cancer patients, aged 64 ± 10 (42-77) years, were included in the study. Eight patients presented with colorectal cancer, 5 patients had gastric cancer, 4 patients had breast cancer, 2 patients had thyroid cancer, 2 patients had cancer of the esophagus and one patient each had cancer of the pancreas and gastric leiomyosarcoma. Eight subjects, aged 58 ± 11 years, presenting for elected operation for inguinal hernia

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served as controls.

In all the studied subjects serum sample was taken before any operative treatment and stored in aliquotes at −50℃.

Neopterin determination

Serum neopterin was determined using a RIA kit (generously provided by Henning, Berlin, Germany) as described (13).

ACE measurement

ACE was measured by a colorimetric method (14). Fifty µl of the serum sample was incubated with 100 µl 20.5 mmol/l hippuryl-glycyl-glycine (Sigma, St. Louis, USA) at 37℃. After 30 minutes, 100 µl 0.33 mol/l sodium tungstate (Lachema, Brno, Czech Republic) and 100 µl of 0.33 mol/l sulfuric acid (Lachema) were added. After mixing, 1000 µl of distilled water was added. The solution was then centrifuged (2000×g for 10 minutes), and 750 µl of the supernatant was incubated with 50 µl of 0.1 mmol/l 2,4, 6-trinitrobenzene sulfonic acid (Sigma) and 1000 µl of borate buffer, pH=9.6, (Lachema) at room temperature. After 30 minutes the absorbance was read at 405 nm. A standard curve was constructed using glycyl-glycine (Sigma).

Statistical analysis

The significance of differences was assessed using the Mann-Whitney U test. The correlation was studied by Spearman rank correlation coefficient. The decision on significance was based on p=0.05 significance level.

Results

Serum neopterin was significantly higher in the cancer patients compared to controls (15.4 ± 12.6, range 4.7～60.6 vs 7.2 ± 2.2, range 5～10.3 nmol/l, p<0.02). On the other hand, ACE activity has not been significantly different (2.6 ± 2.1, range 0.2～8.7 vs 3.4 ± 1.1, range 1.6～5.4 µkat/l for cancer patients and controls, respectively). No correlation could be observed between serum neopterin and ACE levels (Figure 1). Only 3 out of 23 patients had ACE activity higher than the normal range, defined as the mean of the controls+2 standart deviations.

Discussion

As expected from previous studies (5,10,11,12), serum neopterin was significantly higher in cancer patients. On the contrary, ACE was not elevated.

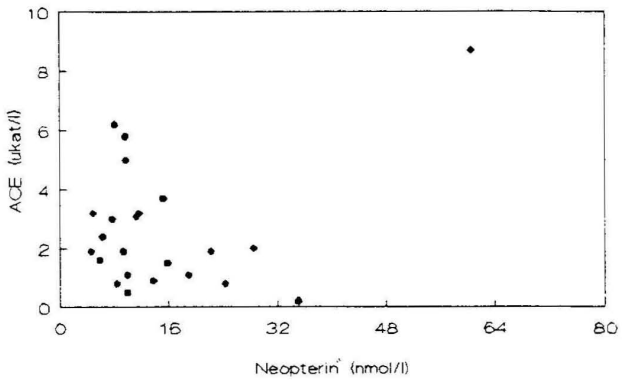

Figure 1. Serum neopterin and ACE in cancer patients (rs= −0.1869, not significant).

ACE is an established marker in sarcoidosis (1,2). Besides this disease, an increase in ACE was reported in a variety of disorders, including HIV infection (3), ulcerative colitis (4), diabetic angiopathy (15) and chronic fatigue syndrome (16). An increase in ACE activity could be also produced in experimental inflammation (17). While in sarcoidosis ACE is thought to be produced by macrophages (1), the cellular source in other mentioned conditions has not yet been unequivocally identified.

Neopterin is an established index of macrophage activation (5). Our results indicate that macrophage activation in cancer patients is not associated with increased serum ACE levels. ACE appears to be elevated only in a small portion of cancer patients.

In conclusion, serum ACE does not seems to be elevated in cancer patients. Macrophage activation in cancer patients, as reflected by increased serum neopterin, is not accompanied by similar rise in ACE. Increased ACE production by macrophages appears to be peculiar to some conditions, notably sarcoidosis, while an elevation in serum ACE in other disorders might come from endothelial cells, but increased ACE, unlike neopterin, does not seem to be a universal marker of macrophage activation.

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Dedicated to Professor Dr. Helmut Wachter on occasion of his 65th birthday.

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Immune Activation and HIV-1 Viraemia in an Asymptomatic Paediatric Patient

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Summary

Immune stimulation due to secondary infection may enhance HIV replication and viraemia, since cellular activation of CD4+ T-cells is required for viral replication. We describe a case of an 18 months old child, exposed intrauterinely to human immunodeficiency virus type 1 (HIV-1). During a period of various infections, which was followed by transient viraemia, decrease of CD4+ T-lymphocytes and increase of neopterin concentrations was registered. Disease progression may have occured due to activation of latent HIV-1 in CD4+ T-lymphocytes/macrophages by secondary infections.

Key words: Immune activation, Human immunodeficienjcy virus, Neopterin

Introduction

The outpatient clinic of the paediatric university hospital in Innsbruck, Austria, cares for 11 children infected with human immunodeficienjcy virus type 1 (HIV-1) aged between 18 months and 16 years. Seven of them were infected by vertical transmission and four received contaminated blood products before 1985 when blood donations have not been tested for HIV antibodies and neopterin concentrations.

HIV-1 infects not only CD4+ T-lymphocytes, but also cells of the monocyte/macrophage lineage expressing the CD4 surface molecule (1). It has been assumed that immune stimulation due to secondary infections may trigger viral replication (2,3), which results in viraemia and death of CD4+ T-lymphocytes.

Antigen-stimulated T-lymphocytes produce high amounts of interferon-γ, which is the main inducer of neopterin release from human macrophages in vitro. Neopterin has been shown to be a sensitive marker for activation of cell mediated immunity (4) also in vivo. The majority of individuals with HIV-1 infection shows increased levels of neopterin, this is true even in early stages of infection. CD4+ T-cell counts and neopterin levels usually correlate well, however, both parameters have been shown to jointly predict disease progression in adults (5,6,7) and in children (8,9).

Changes of CD4+ T-cell counts are less marked in peadiatric patients and decline of CD4+ T-lymphocytes is less significantly correlated with disease progression (9), which contrasts the observations made in adult patients. Although HIV-1 p24 antigenaemia is associated with disease progression (7, 10), p24 antigenaemia is not only rare in asymptomatic adults but also in children.

Case Report

The course of HIV-1 infection in an 18 months old female infant developing viraemia during periods of various childhood infections is described. Being exposed to HIV-1 during pregnancy, the child was spon-

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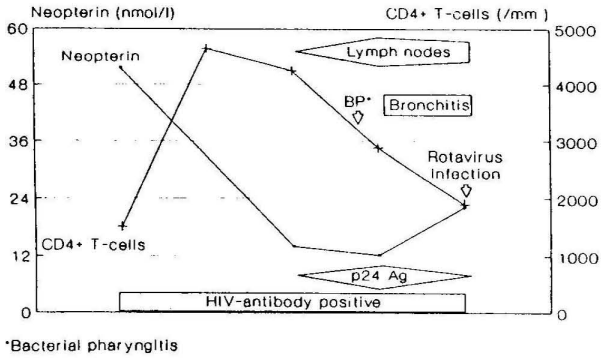

Figure 1. Course of CD4+ T-cell counts, neopterin and HIV-1 -p24 antigen in a child with HIV-1 infection during episodes of secondary infections.

taneously delivered without further complications. Pregnancy was complicated by daily Methadon administration by the HIV-1-seropositive mother within a rehabilitation (anti-drug treatment) program. The newborn showed signs of withdrawal at the third day of life. In the child HIV-1-antibodies have been found from begin to the end of evaluation. HIV-1 nucleic acid was detected by polymerase chain reaction (PCR).

No clinical complications were registered throughout the first year of life. Infancy was normal regarding nutrition, neurological and cognitive development. The child has been undergoing periodic clinical evaluations for 18 months. Blood was screened for concentrations of neopterin, a sensitive indicator of immune activation (radioimmunoassay, Henning, Berlin, Germany), for p24 antigen (Abbott, Chicago, IL) and the CD4+ T-lymphocyte count.

At the age of 14 months the child developed severe bacterial pharyngitis associated with fever (39℃). Oral amoxicilline treatment was able to improve her condition for a period of two weeks only, thereafter the child contracted severe viral bronchitis with heavy cough. When the child recovered from that, she developed enterovirus infection only one month later. While there were no signs of disease progression during the first year of life, the infant developed generalised lymphadenopathy, a remarkable decline in CD4+ T-lymphocytes and viraemia as was documented by p24 antigenaemia.

Neopterin levels constantly decreased during the first year of live, but increased significantly after the enteroviral infection (Figure 1). After complete recovery from viral enteritis, disappearance of both the generalised lymphadenopathy and HIV-1 p24 antigenaemia was also documented. However, neopterin levels remained increased compared to the normal range of healthy children within this age group. Concentrations of beta-2-microglobulin, another valid predictor of progression to AIDS in HIV-1 seropositives, behaved similar to neopterin changes, starting with an increased level, which was followed by a decline and a further rise after secondary infections became manifest (details not shown).

Discussion

Surrogate markers as neopterin and beta-2-microglobulin are well correlated with stage of HIV-1 infection in adult (5,6,7) and paediatric patients (8,9,11, 12). Usually there exists a significant inverse correlation between neopterin levels and CD4+ T-cell counts in adult HIV-1 seropositive patients. In the present case we found increasing levels of serum neopterin associated with decreasing CD4+ T-cell counts. As a whole, neopterin levels appeared to parallel changes of CD4+ T-cell counts in a mirror-image fashion (Figure 1). Most probably immune stimmulation induced by a secondary antigen was involved to accelerate the course of HIV-1 infection from an asymptomatic infection to one with detectable HIV-1 antigen. Due to secondary antigenic stimulation, activated lymphocytes may have triggered HIV-1 replication and disease progression. Enhanced production of neopterin in the child reflects this biological background and supports the view that activation of immune cells was related to disease progression. Similarly, general lymphadenopathy was most prominent during phases of viraemia which was reflected by detectable p24 serum antigen. Based on our observation we postulate that immune activation stimulated multiplication and spread of HIV-1 in the infected child.

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Relationship among Serum hsp65 Antibodies, Neopterin, Autoantibodies and Atherosclerosis

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Summary

A statistical reinvestigation was performed on data from two separate earlier studies, investigating heat-shock protein 65 and neopterin in the same population of subjects examined for the presence of carotid atherosclerosis. Both heat-shock protein 65 and neopterin concentrations are significantly correlated with atherosclerosis, but the correlation between both biochemical analytes is low albeit significant. Neopterin, but not heat-shock protein 65 concentrations were shown to be significantly higher in women with presence of antinuclear antibodies, particularly in women older than 65 years of age. This finding seems to explain earlier observations of raised neopterin concentrations in such older women, irrespective of the presence or absence of atherosclerosis.

Key words: Neopterin; Heat-shock protein 65, Autoantibodies, Atherosclerosis

Introduction

Stress or heat-shock protein (hsp) 65, a highly conserved protein synthesized by almost all cells in response to varying stresses, has been shown to be involved in the pathogenesis of atherosclerosis (1-5). Recently, we reported that serum antibodies to hsp65, but not autoantibodies, were significantly increased in subjects with carotid atherosclerosis in randomly selected normal inhabitants of South Tyrol (4), and these antibodies cross-reacted with a 60 kDa protein homologue present in human atherosclerotic lesions (5).

In this same population, we also found elevated levels of serum neopterin, a pteridine derivative, in subjects with atherosclerosis (6). Both, hsp65 antibodies and neopterin, are considered as indicators of an involvement of immune reactions. Hence, it was deemed of interest to investigate if a relationship exists between these two variables in atherosclerosis.

Here we report a short synopsis of the data of both independent studies (4,6), aimed particularly at studying the mutual correlation between hsp65 antibodies and neopterin concentrations, and at investigating whether presence versus absence of other autoantibodies determined in these sera (4) exerts an influence on neopterin levels.

Methods

As explained in the Introduction, data from two

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earlier independent studies on the same population (4,6) were included in this investigation. Subjects were from a community based study (Bruneck Ischemic Heart Disease and Stroke Prevention Study) conducted in the community of Bruneck (province of Bozen/Alto Adige, Italy). Total study population (n = 1000) was selected as a 21% random sample of all inhabitants aged between 40 and 79 years, whereby men and women were selected equally for inclusion into the study on the basis of an age stratified random sampling strategy.

Complete data on hsp65 antibodies, neopterin and autoantibodies, i.e. antinuclear antibodies (ANA), antibodies to thyroglobulin and thyroid microsomal antigens, and rheumatoid factors, were available for 541 subjects (259 female, 282 male), aged 50 through 80 years.

In all individuals, extensive physical and laboratory examinations were performed, and carotid atherosclerosis was assessed by examination of extracranial carotid arteries using a duplex ultrasound system (UM8 Advanced Technology Laboratories, Bothel, WA) with a 10 MHz imaging probe and a 5 MHz Doppler. A plaque scoring system was used for quantifying the extent of carotid atherosclerosis. This score was calculated by adding the maximum thickness of atherosclerotic plaques (in mm) on the near and far walls at four imaging sites of both carotid arteries, with plaques being defined as echo structures enchoraging into the lumen of the vessel.

Details regarding recruitement of subjects as well as physical and laboratory determinations are reported in references #4 and #6.

Statistical analysis of data was performed using Spearman rank correlation analysis and Mann-Withney U test for comparing distributions of laboratory data in patients with, or without presence of the autoantibodies studied.

Results

Correlation between atherosclerosis score, hsp65 and neopterin

Serum concentrations of both hsp65 antibodies and neopterin significantly correlated with the occurrence and severity of carotid atherosclerosis : Spearman rank correlation coefficient r_s between hsp65 and score was 0.19 ($P<0.0001$), and between neopterin and score, $r_s=0.39$ ($P<0.0001$). Both laboratory variables showed stronger correlation with score in men (hsp65, $r_s=0.23$; neopterin, $r_s=0.47$) than in women (hsp65, $r_s=0.17$; neopterin, $r_s=0.32$).

The correlation between hsp65 antibodies and neopterin levels is weak albeit significantly different from zero ($r_s=0.19$, $P<0.0001$). In contrast to the mutal correlation between score, and neopterin and hsp65, however, the direct correlation between neopterin and hsp65 was stronger in women ($r_s=0.22$) than in men ($r_s=0.16$).

Effect of other autoantibodies on neopterin and hsp65

When studying potential effects of other autoantibodies on neopterin and hsp65 concentrations, there was only one striking result : neopterin but not hsp65 concentrations were significantly higher in subjects with high titers (>1:50) of ANA (Mann-Whitney $U=10499$, $P=0.0091$). No correlations were found between hsp65 and neopterin on the one hand, and the other autoantibodies studied on the other hand (details not reported).

Closer inspection showed that ANA effects on neopterin were significant only in women ($P=0.029$), not in men ($P=0.22$). Furthermore, in women there was a significant association between ANA and age, women above 65 years showing a significantly higher frequency of ANA ($P=0.0004$) presence than women below this age. In men, no such correlation was detectable.

Discussion

Both neopterin and hsp65 showed significant correlations with presence of carotid atherosclerosis, as was described earlier (4,6,7). The correlation between both indicators of immune activation, however, is very weak albeit significant due to the relatively large number of individuals investigated. The small correlation coefficient between hsp65 and neopterin levels suggests that both analytes throw light on different aspects of cellular immune mechanisms.

The most significant finding of the present re-investigation of these earlier data is the strong and unexpected effect on neopterin of the presence of ANA, particularly in women. This observation seems particularly interesting since it may provide an explanation for the striking observation in our earlier paper (6) that neopterin was not only significantly correlated with atherosclerosis in older men and in younger men and women, but was also elevated in older women (above 65 yr) without detectable atherosclerosis. It was speculated (6) that other inflammatory processes could be the underlying cause for this peculiarity. The present finding of a significant positive correlation between neopterin elevation and occurrence of ANA specifically in older women seems to substantiate this concept.

In conclusion, neopterin and hsp65 antibodies, positively correlated with atherosclerosis, may reflect different aspects of immune reactions in the pathogenesis and/or course of carotid atherosclerosis. The stronger correlation between atherosclerosis score and neopterin as compared to hsp65 antibodies, may be partially caused by common correlation of these both variables with other factors such as, e.g., age, sex and presence of ANA.

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