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J. Nat. Prod., **1991**, 54 (4), 959-966• DOI: 10.1021/np50076a006 • Publication Date (Web): 01 July 2004

Journal of Natural Products
Vol. 54, No. 4, pp. 959-966, Jul-Aug 1991

CHEMICAL CHARACTERIZATION AND PHARMACOLOGICAL ACTIVITY OF NAZLININ, A NOVEL INDOLE ALKALOID FROM *NITRARIA SCHOBERI*[1]

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ABSTRACT.—Nazlinin [**1**], a novel indole alkaloid with serotonergic activity, has been isolated together with serotonin and tryptamine from *Nitraria schoberi* using a bioassay-guided fractionation, and its structure has been confirmed by eims and nmr techniques. The contractile and relaxing effects of nazlinin on blood vessels are compared with those of the structurally related serotonin.

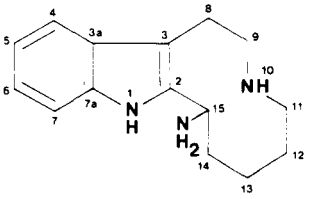
Previous studies on *Nitraria schoberi* L. (Zygophyllaceae), a plant that is widely distributed all over Asia, the Middle East, and Australia, have mostly centered on the isolation and structure elucidation of its alkaloids (1–8). A series of alkaloids, including nitramine, nitraramine, nitraroxine, nitraraine, dihydronitraraine, nitramidine, nitrarine, schoberine, and schoberidine, have been characterized.

Because crude MeOH extracts of *N. schoberi* showed serotonin-like activity in in vitro bioassay tests, we thought it was relevant to examine the components responsible for this activity in more detail. Characterization of novel serotonin-like substances is still of continuing interest because it may provide pharmacological tools for the study of serotonin receptor subtypes in mammalian systems.

Investigation of the bioactive extract has led to the isolation of known biologically active indole alkaloids, tryptamine and serotonin, and a novel indole alkaloid, nazlinin. In this study, we report the isolation, structure elucidation, and pharmacological characterization of these indole alkaloids with serotonergic activity.

RESULTS AND DISCUSSION

Chemical studies on the aerial parts of *N. schoberi* carried out by Russian scientists have resulted in the structure elucidation of a variety of alkaloids (2–8). In the present study, plant extracts of *N. schoberi* were investigated by a bioassay-based approach.



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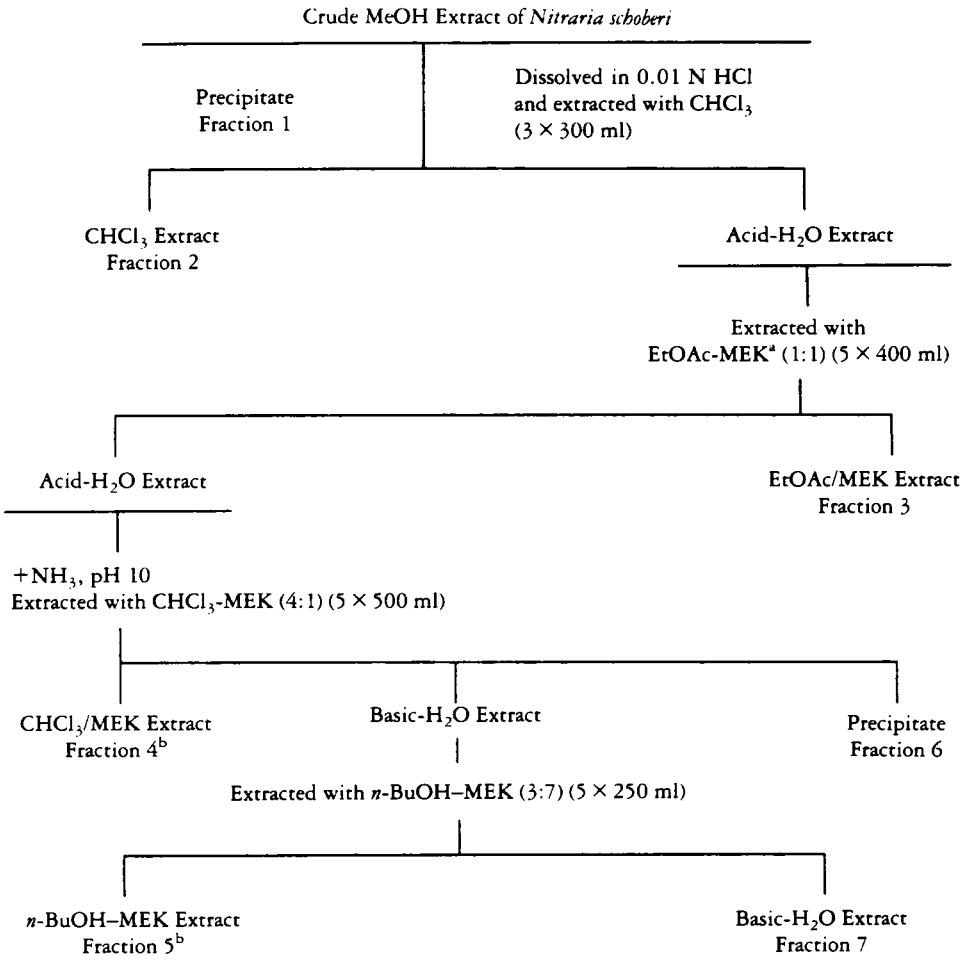
[1]Preliminary results of this work were presented in poster form at the 37th Annual Congress on Medicinal Plant Research in Braunschweig, Germany, 5–7 September 1989 [abstract in *Planta Med.*, **55**, 605 (1989)] and at the Satellite Symposium on EDRF and Related Substances in Antwerp, Belgium, 28–30 June 1990 [abstract in *Arch. Int. Pharmacodyn.*, **305**, 260 (1990)].

Using bioassay-guided fractionation, serotonin-like compounds were isolated, and their structures were identified and their pharmacological activities characterized.

For the detection of serotonin-like activity in crude extracts, fractions, and isolated compounds, the isolated organ superfusion technique was applied. This technique was previously shown to be useful for the detection of prostaglandin-like activity in onion extracts (9).

Biological screening of crude extracts indicated that the MeOH extract showed serotonergic activity. This extract was subjected to further fractionation. Details about the extraction and the isolation of tryptamine, serotonin, and the novel indole alkaloid, nazlinin, are given in Scheme 1 and in the Experimental section.

Nazlinin [**1**] was found to have a molecular formula of $C_{15}H_{21}N_3$ by hreims. The presence of an indolic moiety was indicated by its ir absorptions near 1455 and 1621 cm^{-1} and by the signals at *m/z* 130 and 144 in its ei mass spectrum (10). The spin echo Fourier transform (SEFT) and off-resonance ^{13}C-nmr spectra indicated the presence of



SCHEME 1. Extraction of the biologically active crude MeOH extract of *Nitraria schoberi*.

[a]MEK = methyl ethyl ketone.

[b]Fractions displaying serotonin-like bioactivity.

six aliphatic CH_2 groups, one aliphatic CH group, four aromatic or unsaturated CH groups, and four quaternary aromatic or unsaturated C atoms. The ^1H- and ^{13}C-nmr spectra showed a resonance pattern that could be attributed to a 2,3-disubstituted indole system (11). The COSY spectrum (Figure 2), showed a CH_2-CH_2 and a CH_2-CH_2-CH_2-CH_2-CH system.

All the nmr data are in agreement with the proposed structure. The position of the primary amine group was confirmed by ^{13}C chemical shift calculations and supported by eims data. The formation of the base peak at *m/z* 171 with an elemental composition of $C_{11}H_{11}N_2$ can be rationalized by a straightforward simple fragmentation mechanism, involving an α cleavage and expulsion of a neutral $(CH_2)_4NH_2$ moiety (Scheme 2). According to the COSY nmr data, the primary amine group could also be at C-11. However, in this case, the formation of the abundant ion at *m/z* 171 formed by eims cannot be readily explained.

Both serotonin and nazlinin contracted coeliac and mesenteric arteries. The doses injected on top of the cascade were 1, 10, 30, and 100 ng for serotonin and 1, 10, 30, and 100 μg for nazlinin. A clear dose-response relationship was found within this dose range. At the end of the experiment, 1 μg serotonin was injected. The contraction corresponding to this dose was taken as 100%, and all the other contractions were related to this 100% value. This method resulted in ED_{50} values as given in Figure 2 (nazlinin) and Figure 3 (serotonin).

ED_{50} values for nazlinin were 111 ± 5 nmol (coeliac artery) and 70 ± 5 nmol (mesenteric artery). Serotonin had an ED_{50} value 550 to 700 times lower than that of

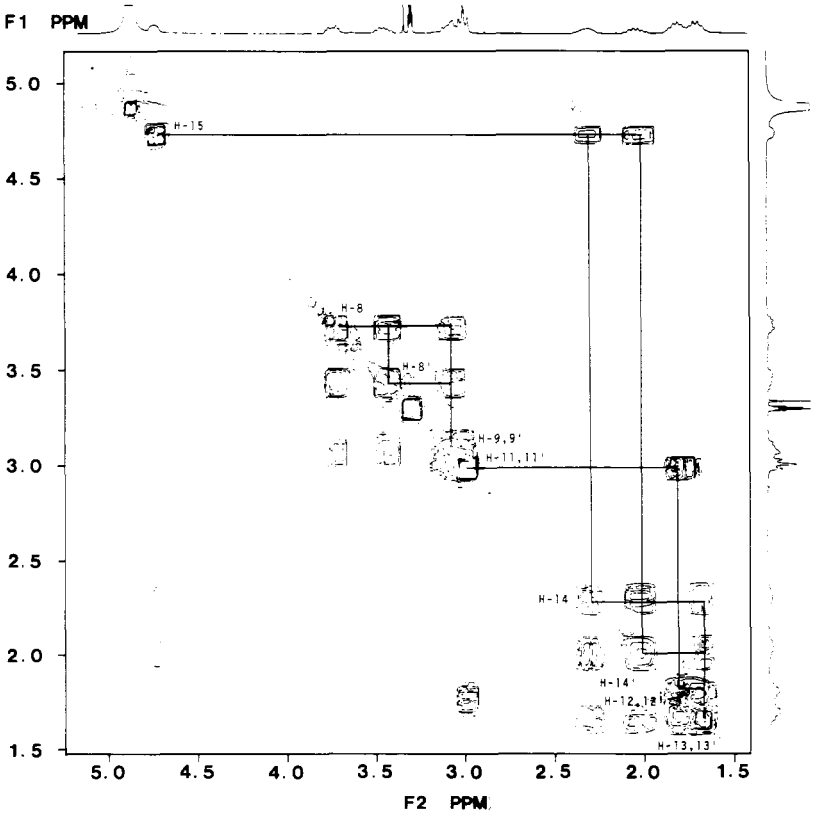
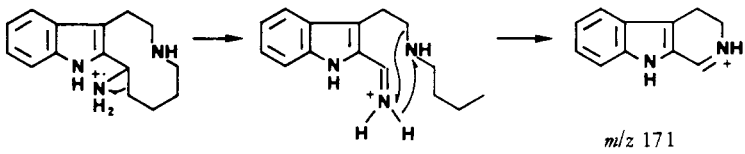


FIGURE 1. ^1H-COSY spectrum of nazlinin [**1**].



SCHEME 2. Formation mechanism for the base peak at *m/z* 171 formed by eims.

nazlinin on mesenteric and coeliac arteries, respectively. Serotonin gave also higher contractions than nazlinin. Maximal contractions were calculated according to Tallarida and Murray (12). E max values for nazlinin were 71% and 83% for coeliac and mesenteric arteries, respectively, whereas the E max values for serotonin were 110% and 108%, respectively.

Nazlinin had a clear-cut vasorelaxing effect upon aortic rings with intact endothelium. At a dose higher than 40 nmol the relaxing effect changed into a contraction. The vasorelaxing effect was completely abolished by mechanical removal of the endothelium, which was checked by acetylcholine challenge (Figure 4).

The results were qualitatively the same for serotonin: relaxations for lower doses on aortic rings with endothelium and contractions for higher doses and/or rings without endothelium (Figure 5). The vasorelaxing activity of serotonin has been reported in the literature. More particularly, experiments were carried out with coronary artery smooth muscle (13–16). $PGF_{2\alpha}$ contractions were shown to be useful to make the relaxations even more pronounced (17). It is not clear from our experiments whether the nazlinin-

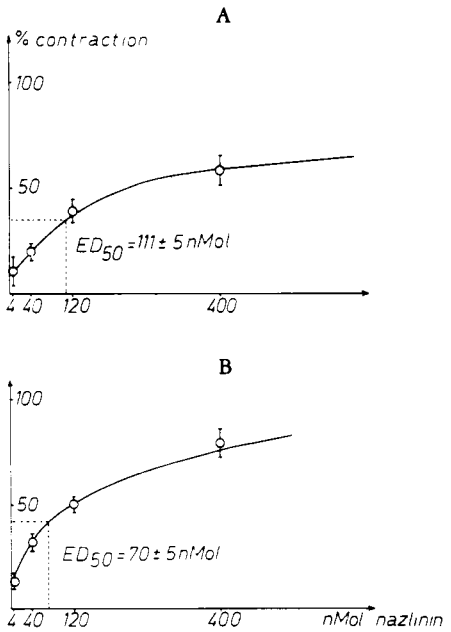


FIGURE 2. Nazlinin-induced contractions of coeliac (A) and mesenteric (B) arteries expressed as percentage of the contraction obtained with 1 μg serotonin. Maximal contractions were calculated using the double-reciprocal plot. Each point represents the mean ± SEM of at least six experiments.

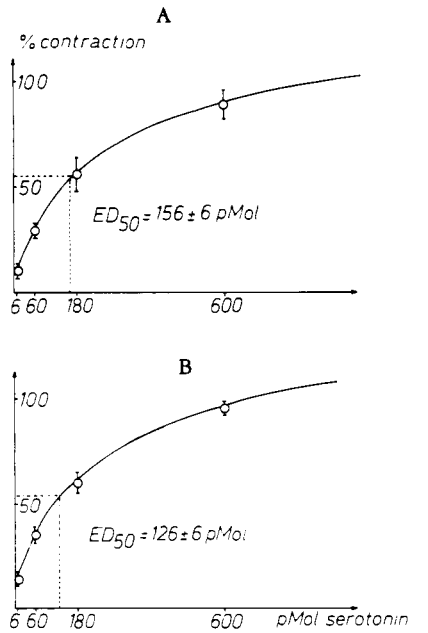


FIGURE 3. Serotonin-induced contractions of coeliac (A) and mesenteric (B) arteries expressed as percentage of the contraction obtained with 1 μg serotonin. Maximal contractions were calculated using the double-reciprocal plot. Each point represents the mean ± standard error of the mean of at least six experiments.

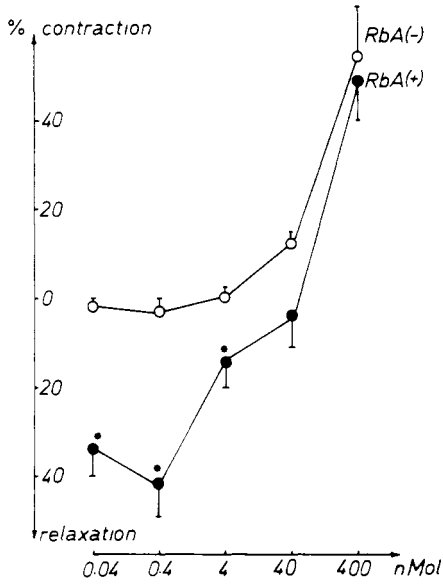


FIGURE 4. Dose-response curves obtained with nazlinin on rabbit aortic rings with [RbA(+)] and without [RbA(−)] endothelium. Contractions and relaxations are expressed as percentage of the contraction obtained with $PGF_{2\alpha}$ (10^{-6} M). Each point represents the mean ± SEM of at least eight experiments. $*p < 0.05$ (Student's *t*-test)

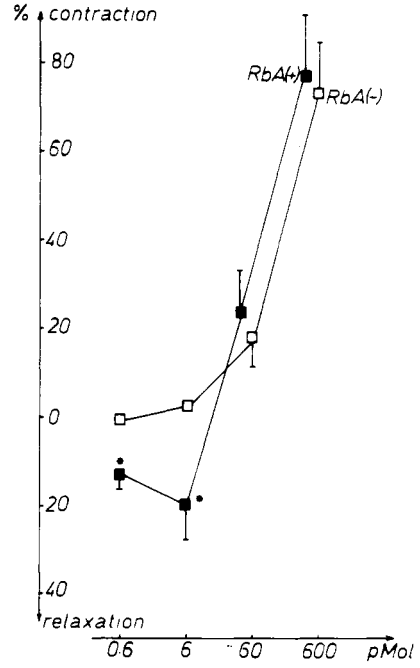


FIGURE 5. Dose-response curves obtained with serotonin on rabbit aortic rings with [RbA(+)] and without [RbA(−)] endothelium. Contractions and relaxations are expressed as percentage of the contraction obtained with $PGF_{2\alpha}$ (10^{-6} M). Each point represents the mean ± SEM of at least eight experiments. $*p < 0.05$ (Student's *t*-test)

and serotonin-induced relaxations are due to the release of endothelium-dependent relaxing factor, for which transfer of the vasorelaxing agent is required in order to identify it (18). The nature of the blood vessels has been shown to play an important role in their response to vasoactive agents (19). Despite this lower sensitivity, aortic rings are a useful experimental tool for the detection of endothelium-dependent vasorelaxations. Ketanserin (100 nM), a serotonin-2 receptor blocker, did not appear to affect the nazlinin-induced relaxations while significantly inhibiting the contractions of higher doses on rings with endothelium (Figure 6).

Other antagonists such as indomethacin (cylooxygenase inhibitor; 5×10^{-6} M), prazosin or phentolamine (α-adrenoceptor antagonist; 10^{-6} M), propanolol (β-adrenoceptor antagonist; 10^{-6} M), and atropine (muscarininc receptor antagonist; 10^{-6} M) did not antagonize the vasorelaxing effect. A more fundamental study, in which the nature of nazlinin-induced relaxations is examined, is currently in progress. More particularly, a series of selective serotonin agonists and antagonists are being evaluated in order to determine the types of serotonin receptors involved (20).

EXPERIMENTAL

GENERAL CHEMICAL PROCEDURES.—*Chromatography*.—Droplet counter current chromatography (dccc) was carried out on a Model A instrument of Tokyo Rikakikai, which was equipped with 300 glass columns of 40 cm length and 3 mm i.d. The solvent system $CHCl_3$–MeOH–*n*-PrOH–H_2O (5:6:1:4) was used in the ascending mode, and fractions of 12 ml were collected at a flow rate of 40 ml/h. Analytical tlc

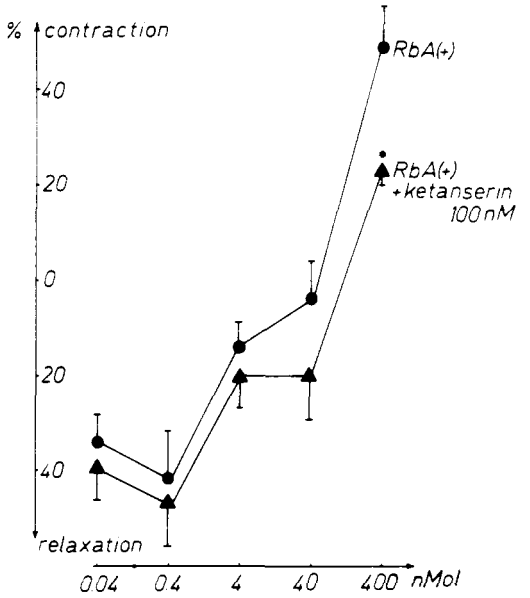


FIGURE 6. Effect of ketanserin (100 nM) on nazlinin-induced contractions and relaxations of rabbit aortic rings with endothelium [RbA(+)], expressed as percentage of the contraction obtained with $PGF_{2\alpha}$ (10^{-6} M). Each point represents the mean ± SEM of at least six experiments. $*p < 0.05$ (Student's *t*-test)

was performed on precoated Si gel plates (Kieselgel $60F_{254}$, 0.25 mm, Merck) using either EtOAc-HCOOH-H_2O (75:15:10) (System A) or EtOAc-iPrOH-NH_3 (25%) (55:30:15) (System B) as developing systems. Visualization of alkaloids was done by uv detection at 254 and 366 mm and spraying with Dragendorff's reagent. Specific detection of serotonin was carried out by exposure to iodine vapors and spraying with ninhydrin reagent. Sephadex LH-20 (Pharmacia) was used for cc.

Spectroscopy.—Uv spectra were obtained on a Beckman Lambda 5UV instrument. Ir spectra were recorded on a Brüker 113V FT spectrometer. Mass spectra were obtained on a VG7OSEQ instrument using direct introduction and electron impact ionization. High resolution measurements were performed at a resolution of 10,000 using perfluorokerosene as reference. ^1H and ^{13}C nmr spectra were recorded on a JEOL JNM-FX-200 instrument at 199.50 and 50.10 MHz, respectively. For the assignment of the ^{13}C-nmr signals, the SEFT multipulse sequence was used, with an interval time of 7 msec ($1/J$), yielding positive signals for CH and Me groups and negative signals for quaternary C atoms and CH_2 groups, because for both groups of resonances a phase difference of 180° exists. With an interval time of 3.5 msec ($1/2J$), SEFT yielded only signals arising from quaternary carbons (21). An off-resonance ^{13}C-nmr spectrum was also recorded. A 1D ^1H-nmr spectrum was also recorded. A 1D ^1H-nmr spectrum and a 2D homonuclear (^1H) COSY spectrum were recorded on a Varian XL-300 instrument at 300 MHz. The samples were dissolved in MeOH-d_4 (99.5 atom % D). Chemical shifts are reported in ppm (δ-scale) relative to TMS (0 ppm).

PLANT MATERIAL.—*N. schoberi* was collected in May 1987 near the village of Sereflikoçhisar in the province of Ankara, Turkey. Plant specimens were deposited in the herbarium of the University of Ege. The epigeal part of the plant material was dried without exposure to direct light and powdered.

CRUDE EXTRACTS.—In order to screen the biological activity of *N. schoberi*, a percolation procedure was applied using different solvents of increasing polarity. The dried and powdered plant material (1.28 kg) was percolated with $CHCl_3$ (3 liters), EtOAc (3 liters), and MeOH (3 liters). Each percolate was dried by rotary evaporation under reduced pressure and at a temperature not exceeding 40°. The residues were kept at −20° until analyzed. Biological screening of the extracts indicated that only the MeOH extract (yield 16% dry wt) showed serotonin-like activity in the in vitro bioassay tests.

FRACTIONATION OF THE BIOACTIVE MeOH EXTRACT.—The MeOH extract was fractionated by a conventional extraction procedure, and seven fractions were obtained (Scheme 1). Serotonin-like activity was mainly localized in two fractions, i.e., fractions 4 and 5, which were further separated. Fraction 4 was

subjected to dccc, and fractions were monitored by analytical tlc using solvent system A. Fractions showing an identical pattern on tlc were combined in a total of seven fractions. Serotonin-like activity was found for the first two, i.e., fractions 4.1 and 4.2. Fraction 5 was fractionated by cc on Sephadex LH-20 using gradient elution (50% aqueous MeOH to 100% MeOH), and analytical tlc was performed with solvent system A. A total of six fractions were obtained, and serotonin-like activity was found for Fraction 5.4.

PURIFICATION OF COMPOUNDS WITH SEROTONERGIC ACTIVITY AND CHEMICAL IDENTIFICATION OF TRYPTAMINE AND SEROTONIN.—Fractions 4.1 and 4.2 were further separated by cc on Sephadex LH-20 using gradient elution (50% aqueous MeOH to 100% MeOH). Fraction 4.1 was chromatographed with MeOH-H_2O (7:3), whereas Fraction 4.2 was developed with MeOH–*n*-BuOH (4:1). Analytical tlc was performed with solvent system A. Fraction 4.1 resulted in two fractions, and bioactivity was detected in the second fraction, i.e., fraction 4.1.2. This fraction was dissolved in a minimal volume of EtOAc, and precipitation with hexane yielded nazlinin [**1**] (20 mg; yield 0.002% dry wt). Fraction 4.2 afforded three fractions, and bioactivity was found for the third fraction, i.e., fraction 4.2.3. This fraction was analyzed by tlc using solvent system B. Serotonin and tryptamine were used as standards. The substance present in Fraction 4.2.3 co-chromatographed with tryptamine. Further analysis by eims confirmed its identity as tryptamine. Fraction 5.4 was analyzed by 2D tlc using solvent system B for the first development and system A for the second one. Serotonin and tryptamine were applied as standards. The substance present in Fraction 4 co-chromatographed with serotonin and gave the typical color reactions of serotonin. The identity of serotonin was confirmed by its pharmacological profile in the bioassay tests and by eims.

CHEMICAL CHARACTERIZATION OF NAZLININ [**1**].—Nazlinin was isolated as a white amorphous powder, I.U.P.A.C. name 1,2,3,4,5,6,7,8-octahydro-azecino[5,4-*b*]indole-8-amine: uv λ max (MeOH) 271, 280, 288 nm; ir ν max (film) 745, 996, 1053, 1114, 1133, 1455, 1621, 2937, 3370 cm^{-1}; eims *m/z* [**M**]$^+$ 243 (74%), 225 (13), 197 (18), 184 (13), 171 (100), 169 (27), 154 (22), 144 (31), 130 (17), 115 (14); hreims *m/z* 243 ($C_{15}H_{21}N_3$) (mass measured 243.1743, mass calculated 243.1735), 171 ($C_{11}H_{11}N_2$) (mass measured 171.0903, mass calculated 171.0922); ^{1}H nmr (300 MHz, MeOH-d_4, TMS) 1.70 (2H, m, H-13, H-13′), 1.83 (2H, m, H-12, H-12′), 2.03 (1H, m, H-14), 2.30 (1H, m, H-14′), 3.00 (2H, m, H-11, H-11′), 3.10 (2H, m, H-9, H-9′), 3.45 (1H, m, H-8), 3.72 (1H, m, H-8′), 4.72 (1H, m, H-15), 7.05 (1H, dt, J = 7.5 Hz, J = 1.5 Hz), 7.15 (1H, dt, J = 7.5 Hz, J = 1.5 Hz, H-5, H-6), 7.38 (1H, d, J = 7.5 Hz), 7.48 (1H, d, J = 7.5 Hz, H-4, H-7); ^{13}C nmr (50.10 MHz, MeOH-d_4, TMS) 19.5 (t, C-13), 23.2 (t, C-8), 28.2 (t, C-12), 32.8 (t, C-14), 40.4 (t, C-9), 43.0 (t, C-11), 54.6 (d, C-15), 107.4 (s, C-3), 112.4 (d, C-7), 119.0 (d, C-4), 120.6 (d, C-6), 123.5 (d, C-5), 127.4 (s, C-3a), 129.8 (s, C-2), 138.3 (s, C-7a).

BIOASSAY PROCEDURES.—Detection of serotonin-like activity was carried out by the isolated organ perfusion technique, for which rabbit aorta, coeliac and mesenteric arteries were used. The system was adapted in order to detect the biological activity of crude extracts, fractions, and isolated compounds.

For the isolation of the tissues, rabbits of either sex (2.5–3.5 kg, Dendermondse Witte) were killed by a blow on the head and bled, and the aorta and coeliac and mesenteric arteries were removed. Coeliac and mesenteric arteries were cut spirally and fixed on one side of the cascade, whereas aortic tissue was prepared and fixed in two separate ring preparations, one with denuded endothelium on top of the system and the other with intact endothelium at the bottom. A load of 3 g was applied to each tissue. The isolated organs were superfused by means of a Gilson Minipuls II roller pump at a rate of 5 ml/min. The superfusion fluid, oxygenated by carbogen (5% CO_2 + 95% O_2) and thermostatically controlled at 37°, was Krebs solution with the following composition in g/ml (mM): NaCl 6.9 (118), KCl 0.35 (4.7), $CaCl_2 \cdot 2H_2O$ 0.55 (2.5), K_2HPO_4 0.16 (1.2), $MgSO_4 \cdot 7H_2O$ 0.29 (1.17), glucose 2.0 (11.1), $NaHCO_3$ 2.1 (25.0). When necessary, one or more of the following antagonists were added to the Krebs solution or introduced by means of a perfusion pump via a by-pass: ketanserin tartrate (Janssen Pharmaceutica), propranolol HCl (Sigma), prazosin (Pfizer), phentolamine (Regitine, Ciba-Geigy), atropine sulfate (Merck), and indomethacin (Sigma). In order to test relaxing effects dependent or independent of vascular endothelium, ring preparations were contrated by $PGF_{2\alpha}$ (Dinolytic, Upjohn), which was injected by another perfusion pump at a final concentration of 10^{-6} M. The contractions and relaxations of the isolated organs were auxotonically detected by Harvard smooth muscle transducers and recorded with two-channel recorders.

All extracts, fractions, and isolated compounds were tested qualitatively on the cascade superfusion system throughout the separation and isolation procedures. Exogenous serotonin creatinine sulfate complex (Sigma) was applied to evaluate the sensitivity of the isolated organs and to compare the biological responses of the samples under trial. Aortic rings were tested for the presence of functional endothelium by exposure to 1 μg acetylcholine (Ach). Intact rings relaxed to at least 60% of the $PGF_{2\alpha}$-induced contraction, whereas endothelium-denuded rings showed a contraction of about 5%. Rings not meeting these criteria were not used. Stock solutions of agonists and antagonists were prepared weekly in distilled H_2O

and were diluted in Krebs solution before superfusing them over the cascade. Indomethacin and serotonin solutions were made up daily in 1 M TRIS buffer (pH 8; 37°) and distilled H_2O, respectively, and diluted with Krebs solution.

After the purification and structure elucidation of nazlinin, its pharmacological activity profile was examined. Details about the serotonin-like properties of nazlinin are given in Results and Discussion.

ACKNOWLEDGMENTS

The authors thank G. Mannes (Agfa Gevaert, Mortsel, Belgium) for recording the 300 MHz nmr spectra and W. Lauwers (Janssen Pharmaceutica, Beerse, Belgium) for help with the I.U.P.A.C. nomenclature of nazlinin. L.A.C. Pieters and M. Claeys are researchers associated with the Belgian National Fund for Scientific Research. Financial support by the Belgian Government (Grant 87/92-102) and by the Belgian National Fund for Medical Research (Grants 3.0089.87 and 3.0019.89) is gratefully acknowledged.

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Received 8 October 1990