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Distachyasin: A new antioxidant metabolite from the leaves of *Carex distachya*

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Abstract—A novel antioxidant prenylated stilbenoid, distachyasin, has been isolated from the leaves of *Carex distachya*. Its structure has been elucidated on the basis of the spectroscopic characteristics. Bidimensional NMR, and crystallographic data and computational calculations have furnished important data useful for the characterization and the stereochemistry of the molecule. The compound has a tetracyclic skeleton derived from carexane. The compound has been assayed, for the antioxidant activity, by measuring its capacity to scavenge the H_2O_2, nitric oxide, superoxide radical and to inhibit formation of TBARS (thiobarbituric acid reactive species).

Reactive oxygen species (ROS), generated in many bioorganic redox processes, are the most dangerous by products in the aerobic environment. They include the hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^{\cdot}$), the superoxide radical ($O_2^{-\cdot}$), and the nitric oxide (NO). In the last years many evidences correlated ROS to a wide variety of disease states, including cancer, diabetes, cardiovascular diseases, neurodegenerative processes, etc.[1–4] Antioxidants are chemicals that reduce oxidative damage in cells and biomolecules. As some synthetic antioxidants may exhibit toxicity, the search for new bioactive molecular skeleton from natural sources is a very interesting field and recently we have reported the isolation of new antioxidant metabolites from plants.[5]

Carexanes[6,7] are phenolic secondary metabolites isolated from the aerial part of *Carex distachya*,[8] a herbaceous plant, growing in the Mediterranean *macchia*. These compounds originated by the prenylation and successive cyclization of a stilbene precursor. They were believed to be interesting and are mentioned in 'Hot off the press' of Natural Product Reports.[9] It usually has been showed that *Carex* species produce phytoalexinic oligostilbenes,[10,11] constituted by two to four stilbenoid moieties.

In pursuing the study of the hexane extract of *C. distachya* we isolated an unusual polyoxygenated carexane derivative, named distachyasin, which was assigned the tetracyclic structure **1** (Fig. 1).[12]

Its elemental analysis and the pseudomolecular peak at *m/z* 327.1614 (100) in the HR ESI mass spectrum justified the presence of 10 unsaturations in the molecule, according to the molecular formula $C_{20}H_{22}O_4$.

The NMR values indicated substantial modifications to the carexane skeleton. In the downfield, the ^{1}H NMR spectrum, in DMSO, showed several differences with respect to carexanes A–C.[5] Besides the four aromatic H-11–H-14 protons of the D ring, two doublets at δ 5.30 and 6.13 were also evident. In the aliphatic region of

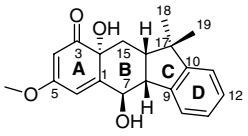

Figure 1. Chemical structure of distachyasin.

Keywords: Distachyasin; Prenyl stilbenoid; *Carex distachya*; Spectroscopic analysis; Crystallographic data; Antioxidant activities.
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Table 1. NMR data of distachyasin (**1**)[a]

Compound	δ ^{1}H	J (Hz)	DQ-COSY (H → H)	δ ^{13}C	DEPT	HMBC (H → C)	HSQC–TOCSY (H → C)	NOESY (H → H)
1	—	—	—	160.4	C	—	—	—
2	—	—	—	70.2	C	—	—	—
3	—	—	—	200.5	C	—	—	—
4	5.30 d	(2.4)	6	95.2	CH	1, 2, 3, 5, 6	4, 6	OCH_3
5	—	—	—	169.9	C	—	—	—
6	6.13 d	(2.4)	4	113.1	CH	1, 2, 4, 5, 7	4, 6	—
7	4.45 d	(7.8)	8	70.6	CH	1, 2, 6, 8, 9, 16	7, 8, 15, 16	14, C7–OH
8	3.08 dd	(7.8, 7.8)	7, 16	54.4	CH	1, 7, 9, 10, 14, 15, 16, 17	7, 8, 15, 16	16, 19
9	—	—	—	143.1	C	—	—	—
10	—	—	—	151.1	C	—	—	—
11	7.15 m		12	121.9	CH	9, 10, 13, 17	11, 12, 13, 14	12, 18, 19
12	7.21 m		11	127.1	CH	11, 14	11, 12, 13, 14	11, 13
13	7.18 m		14	126.5	CH	11, 14	11, 12, 13, 14	12, 14
14	7.50 m		13	124.5	CH	8, 9, 10, 12	11, 12, 13, 14	7, 13
15α	2.25 dd	(13.2, 4.5)	15β, 16	32.5	CH_2	1, 2, 3, 8, 16, 17	7, 8, 15, 16	15β, 18
15β	1.37 dd	(13.2, 10.2)	15α, 16			1, 2, 3, 8, 16, 17	7, 8, 15, 16	15α, 16
16	1.77 m		8, 15α, 15β	47.1	CH	2, 7, 8, 9, 10, 15, 17, 18, 19	7, 8, 15, 16	8, 15β, 19
17			—	44.8	C	—	—	—
18	1.22 s		—	24.7	CH_3	10, 16, 17, 19	—	11, 15α, 19
19	1.05 s		—	30.6	CH_3	10, 16, 17, 18	—	8, 11, 16, 18
OCH_3	3.78 s		—	56.1	CH_3	4, 5, 6	—	4
C2–OH	5.30 br s	—	—	—	—	—	—	—
C7–OH	5.25 br s	—	7	—	—	—	7, 8, 15, 16	7

[a] Data were recorded in DMSO-d_6 on Varian Mercury 300 MHz (^{1}H, ^{13}C) spectrometer (DQ-COSY, TOCSY, HSQC, HMBC, HSQC–TOCSY, ROESY, and NOESY); chemical shifts (δ) were expressed in parts per million with reference to the signal of DMSO (δ 2.49 ppm) for ^{1}H, and to the centre peak of the signal of DMSO (δ 39.5 ppm) for ^{13}C, respectively.

the spectrum were evident a doublet at δ 4.45, a methine proton as a double doublet at δ 3.08, a diasterotopic methylene as two double doublets at δ 2.25 and 1.37, a methine as multiplet at δ 1.77 and two methyl singlets at δ 1.22 and 1.05.

In the spectrum two alcoholic protons, as broad singlets, at δ 5.30 and 5.25 were also evident. This latter showed correlation, in the DQ-COSY experiment, with the doublet at δ 4.45, which was correlated with the double doublet at δ 3.08, which correlated with the methine at δ 1.77, which showed, in turn, cross peaks with the methylene protons Table 1.

The ^{13}C NMR spectrum and the DEPT experiment indicated the presence of three methyls, a methylene, nine methines and seven tetrasubstituted carbons.

The HMBC experiment (Fig. 2) showed correlations between the protons and the carbons of the C and D rings, as registered for the carexanes A–C, but the relationships between the others' nucleus indicated a modification of the molecular skeleton for the A and B rings. In fact, the carbonyl carbon at δ 200.5 showed heterocorrelations with the proton at δ 5.30 and with the methylene protons at δ 2.25 and 1.37. These protons were also correlated with the tetrasubstituted carbons at δ 70.2 and 160.4 and with the methine carbons at δ 47.1 and 54.4. Furthermore, the signal at δ 70.6, bonded to the proton at δ 4.45, showed correlations with the protons at δ 3.08, 1.77, and 6.13. This latter was heterocorrelated with the carbons at δ 70.2, 70.6, 95.2, 160.4, and 169.9 which in turn showed cross peaks with the proton at δ 3.78 and the proton at δ 5.30.

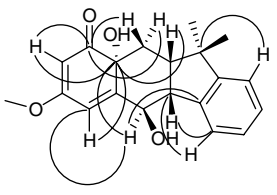

Figure 2. Selected correlation showed in the HMBC experiment.

The above observed HMBC correlations, coupled with two proton spin systems, H-4/H-6, H-11/H-12/H-13/H-14, and C7–OH/H-7/H-8/H-16/H-15α/H-15β, established by TOCSY correlations, suggested the structure of a modified carexane having an α, β, γ, δ unsaturated carbonyl group at C-3 and a hydroxyl group at the C-2 carbon.

Analysis of the HMBC spectrum of **1** also showed obvious correlations from both CH_3-18 (δ 1.22) and CH_3-19 (δ 1.05) to C-10, C-16, and C-17; from H-16 (δ 1.78) to C-2, C-7, C-8, C-9, C-10, C-15, C-17, C-18 and C-19, from H-14 (δ 7.50) to C-8, C-9, C-10, C-12; from H-11 (δ 7.15) to C-9, C-10, C-13, C-17, from H-8 (δ 3.08) to C-1, C-7, C-9, C-10, C-14, C-15, C-16, and C-17; from H-7 (δ 4.45) to C-1, C-2, C-6, C-8, C-9, and C-16.

The absolute configuration of the C-7 carbon was assigned using a modified Mosher method.[13] The positive $\Delta\delta_{R-S}$ values for the H-4 and the H-6 and the negative $\Delta\delta_{R-S}$ value for the H-8 proton indicated a *R* configuration for C-7 carbon. The coupling constants of the H-7 (d, 7.8 Hz), H-8 (dd, 7.8 and 7.8 Hz) were

inadequate to establish the geometry of H-7/H-8/H-16 protons. In fact the *J* values observed could be consistent with both *trans* and *cis* geometry on the basis of computational calculation using molecular mechanic method (MM+) as implemented in HyperChem 7.5. A NOESY experiment showed correlation between the H-7 proton and H-14/C7–OH protons, between the H-8 proton and H-16 and the methyl at δ 1.05. These data suggested a *trans* orientation between the H-7/H-8 and a *cis* orientation between the H-8/H-16 protons. Further NOESY correlations of H-16/H15 (δ 1.37) and the methyl at δ 1.22 with the H-15 proton at δ 2.25 supported the formulated hypothesis.

To confirm the hypothesis and to establish the configuration at the C-2 carbon, X-ray analysis was performed for distachyasin.

Colourless crystals of distachyasin in the form of thin plates were obtained by slow evaporation from chloroform–methanol (1:2) solution at 4 °C.

The X-ray structure[14] of **1** is represented in Figure 3. The torsion angles H(7)–C(7)–C(8)–H(8) and H(8)–C(8)–C(16)–H(16) were −146.4(9)° and −33.9(8)°, respectively, showing *anti*$^{-}$–*gauche*$^{-}$ conformations. So, on the basis of the Mosher's results we assigned the *R*, *R*, *S* for the C-7, C-8, and C-16, respectively. Furthermore, the torsion angles H(7)–C(7)–C(1)–C(6) and O(1)–C(2)–C(1)–C(6) were 126.4(11)° and −96.1(9)°, respectively, in accordance with the *anti*$^{+}$–*anti*$^{-}$ conformations. These data suggested a *cis* orientation among the hydroxyl at the C-2 and the H-7 proton, indicating a *R* configuration for the C-2 carbon.

In Figure 4, the mode of packing of **1** as viewed along the *c* direction is shown. Two molecules of **1** are connected along the *c* axis by two hydrogen bonds that involve the O(3) with the O(4). This arrangement gives rise to a polymeric inclusion structure forming low rows. These rows are arranged in layers of parallel molecules along the *ab* plane. The layers pack in an antiparallel fashion perpendicular to the *c* plane. The rows are held together by a complex intermolecular H-bonds, in which O(3)–H···O(1)–H and O(3)–H···O(4)–H are formed.

Distachyasin was tested for its antioxidant activity. Evaluation of antioxidant activity was carried out using four different methods. Three of these methods estimate radical scavenging activity of investigated substance against the superoxide radical,[15] the pro-oxidant hydrogen peroxide[16] and the nitric oxide;[17] the remaining test evaluates the capacity to inhibit peroxidative processes by measuring formation of TBARS[18] substances. The standard used in all methods was ascorbic acid, a known natural antioxidant, and the results are reported in Figures 5–8. The substances were tested in increasing concentrations (0.1, 0.2, 0.3, and 0.5 mg/ml).

When scavenging of superoxide radical was tested, a linear increase of the activity through the increase of concentration of **1** was observed. The strongest activity was observed at 0.5 mg/ml: it reduced the radical for 60% (Fig. 5). The standard showed an activity of 56% at the same concentration.

Distachyasin showed hydrogen peroxide scavenging activity comparable to the activity exercised by the standard compound (Fig. 6). The molecule was already active at lowest tested concentrations (0.1 and 0.2 mg/ml) whose showed a reductive power on pro-oxidant equal to 32% and 39%, respectively.

The compound **1** scavenged NO radical in a dose dependent manner and showed peculiar activity at highest tested concentrations. It reduced nitrite concentration in the assay media to 10.7% at 0.5 mg/ml. Lower activity was exercised by the standard molecule (Fig. 7).

Although distachyasin showed a weaker activity than ascorbic acid, it inhibited the formation of reactive species to thiobarbituric acid over 59.0% at the concentration of 0.5 mg/ml and inhibiting pattern was carried out in a dose-dependent manner (Fig. 8).

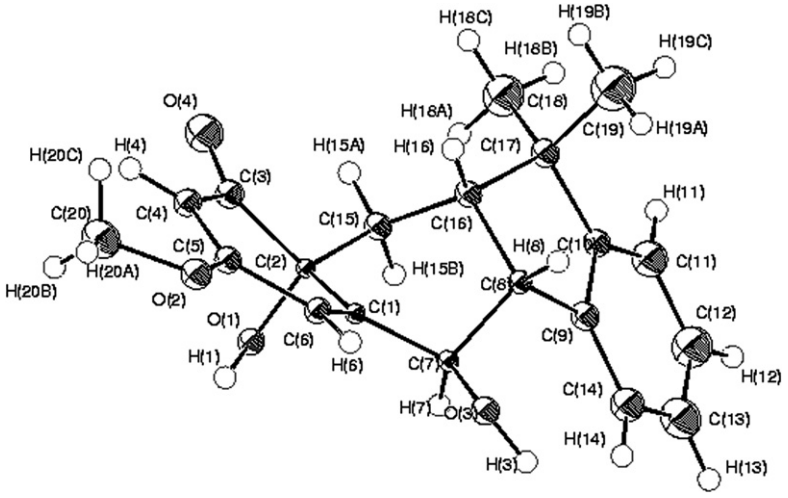

Figure 3. X-ray diffraction structure of **1**, with the numbering of the atom. Displacement ellipsoids are drawn at 50% probability level.

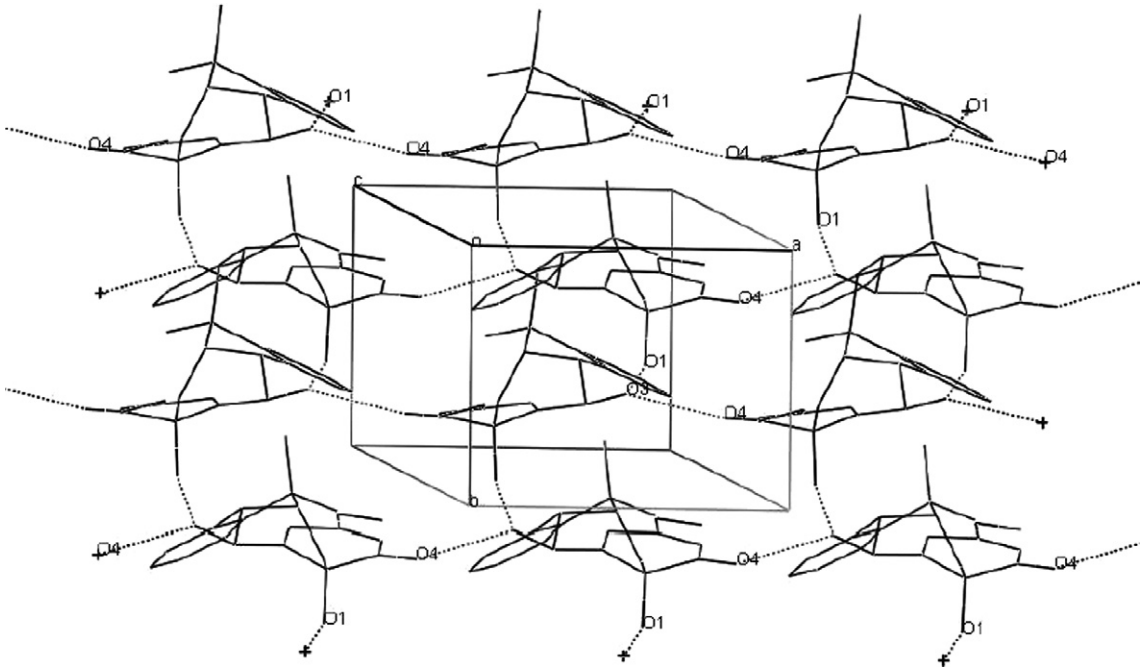

Figure 4. The mode of packing of **1** as viewed along the *c* direction is shown. Hydrogen bonding is represented by dashed line.

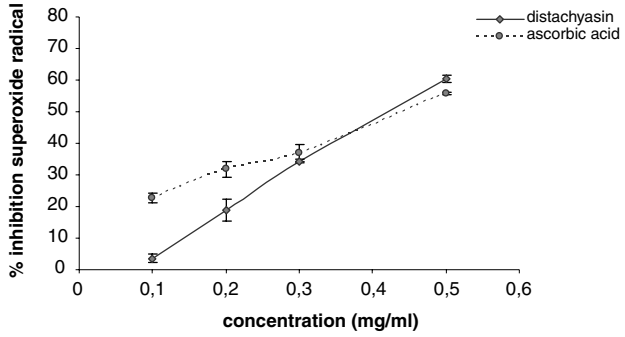

Figure 5. Scavenging activity of superoxide radical of **1**. Values are presented as percentage differences from blank.

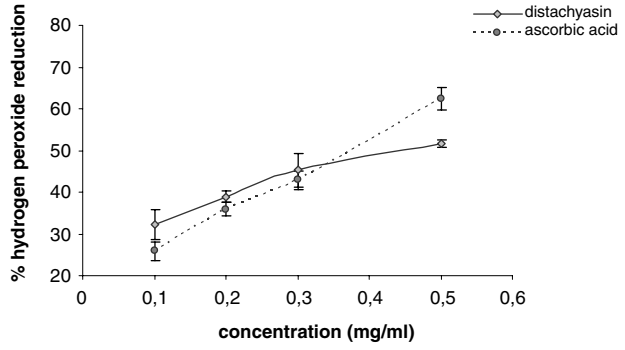

Figure 6. Scavenging activity of hydrogen peroxide of **1**. Values are presented as percentage differences from blank.

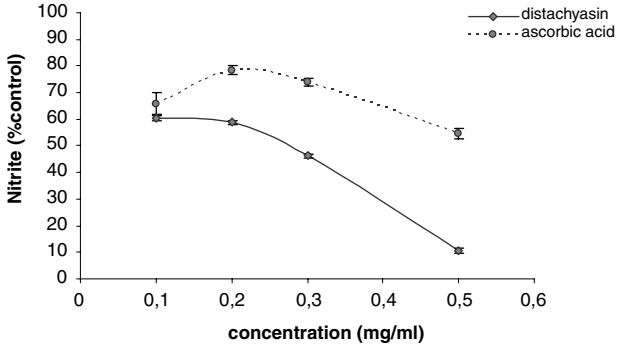

Figure 7. Effect of **1** on the accumulation of nitrite upon decomposition of sodium nitroprusside.

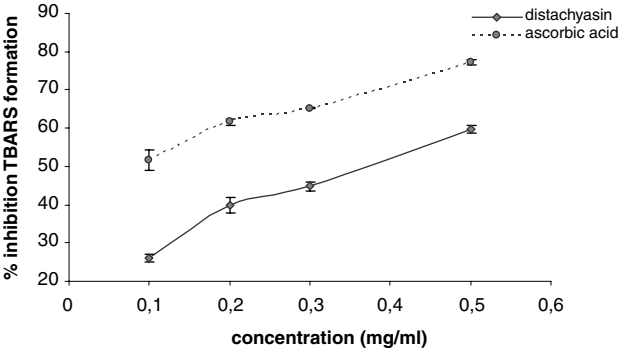

Figure 8. Inhibition of TBARS substances by **1**. Values are presented as percentage differences from blank.

Today many researches focalise their attention to antioxidant activity of secondary metabolites isolated and characterized from natural sources. The results revealed that distachyasin had a significant antioxidant activity.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.08.106.

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12. The hexane extract of *C. distachya* (30 g) was chromatographed on SiO_2 eluting with hexane and EtOAc solutions. Fraction eluted with hexane–EtOAc (3:2) was purified on Sephadex LH-20 [hexane–$CHCl_3$–MeOH (3:1:1)] first and then on RP-8 HPLC [MeOH–MeCN–H_2O (2:2:1)] to obtain pure distachyasin: (18 mg) colorless crystals. $[\alpha]_D^{25}$ +145.0 (*c* 0.28, MeOH); UV λ_{max} (log ε) (MeOH) 283 (3.47), 203 (4.88) nm; HR ESIMS *m/z* 327 $[M+H]^{+\cdot}$ (100), 309 $[M - H_2O+H]^+$ (68). Anal. Calcd for $C_{20}H_{22}O_4$: C, 73.83; H, 6.51. Found: C, 73.45; H, 6.82. ^1H NMR (300 MHz, DMSO-d_6) and ^{13}C NMR (75 MHz, DMSO-d_6): Table 1. The (*S*)-MTPA ester had the ^1H NMR spectral data (300 MHz, CD_3OD): δ 5.97 (2H, d, J = 2.5 Hz, H-6), 5.41 (2H, d, J = 2.5 Hz, H-4), 5.98 (2H, d, J = 9.6 Hz, H-7), 3.83 (6H, s, OMe), 3.50 (obscured, detected by DQ-COSY, H-8), 1.37 (6H, s, H-19), 1.20 (6H, s, H-18). The (*R*)-MTPA ester had the ^1H NMR spectral data (300 MHz, CD_3OD): δ 6.01 (2H, d, J = 2.4 Hz, H-6), 5.47 (2H, d, J = 2.4 Hz, H-4), 6.02 (2H, d, J = 8.8 Hz, H-7), 3.47 (obscured, detected by DQ-COSY, H-8), 3.87 (3H, s, OMe), 1.20 (1H, s, H-19), 1.07 (3H, s, H-18). NMR experiments were recorded at 300 MHz for ^1H and 75 MHz for ^{13}C on a Varian Mercury 300 spectrometer Fourier transform NMR. HR mass spectra were obtained with a ESI-MS Waters 2690-ZMD instrument.
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14. Crystal data for **1**: $C_{20}H_{22}O_4$, M = 326.392, monoclinic, space group $P2_1$, a = 8.44(5) Å, b = 6.91(5) Å, c = 14.75(5) Å, V = 833.(8) Å^3, Z = 2, d = 1.302 g/cm^3, crystal dimensions $0.15 \times 0.14 \times 0.06$ mm was used for measurements on a Nonius MACH3 diffractometer with a graphite monochromator ($\omega - 2\theta$ scans, $2\theta_{max}$) 50.0°, Mo Kα radiation. The total number of independent reflections measured was 2911, of which 1292 were observed ($|F|^2 \geqslant 2\sigma|F|^2$). Final indices: R_1 = 0.0597, wR_2 = 0.1533, S = 0.719, $(\Delta/\sigma)_{max_2}$ = 0.007, $(\Delta/\Delta)_{min}$ = −0.343 e/Å^3, $(\Delta/\Delta)_{max}$ = 0.352 e/Å^3. The crystal structure of **1** was solved by direct method SIR92 (Altomare, A., Cascarano, G., Giacovazzo, C., Guagliardi, A., Burla, M. C., Polidori, G., Camalli, M. *J. Appl. Crystallogr.* **1994**, *27*, 435) and expanded using difference Fourier techniques, refined by the program SHELXL-97 (Sheldrich, G. M. SHELXL97. Program for the refinement of crystal structures. University of Gottingen: Gottingen, Germany, 1997) and the full-matrix least-squares calculations. Crystallographic data for the structure of **1** have been deposited in the Cambridge Crystallographic Data Centre (deposition number: CCDC 605909). Copies of these data can be obtained free of charge via www.ccdc.cam.ac.uk www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB21EZ, UK, fax: (+44) 1223 336 033; or deposit@ccdc.cam.ac.uk).
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nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine was read at 540 nm and referred to the absorbance of standard solution of sodium nitrite treated in the same way with Griess reagent.

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